Short and long term plasticity in mice lacking fyn tyrosine kinase and associated N-methyl-D-aspartate receptor signalling molecules

by

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This work could not have been contemplated without the help of these people, you all know why you appear below, this is a chance for me to say thank you

to Mum and Dad, its all been worth it !

to Jolly for keeping me sane / insane

to Zoë for staying there (!) and showing me the way forward

to John for too much help

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and to The Hartnoll Brothers for all the moments

'The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed'

Albert Einstein

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Abstract

The N-methyl-D-aspartate (NMDA) type of glutamate receptor has important functions in the processes of neuronal development, forms of neurodegeneration, epilepsy, pain and especially in synaptic plasticity and forms of learning and memory. An NMDA receptor dependent form of synaptic plasticity, long-term potentiation (LTP), has been proposed as a cellular correlate of memory. The cellular and molecular mechanisms that underlie this form of synaptic plasticity are at the present time unclear. The NMDA receptor is part of a macromolecular complex that mediates both the localisation of the receptor and its interactions with signalling molecules that activate the intracellular pathways responsible for the long lasting changes in synaptic efficacy. This thesis details results of experiments investigating long and short-term plasticity in mice lacking signalling molecules associated with the NMDA receptor.

The *in vitro* hippocampal slice preparation and extracellular and intracellular electrophysiological recording techniques were used to investigate plasticity in mice lacking the non-receptor tyrosine kinase fyn, focal adhesion kinase (FAK) and the ras guanine nucleotide releasing factor (ras-GRF). Ras-GRF knockout mice displayed normal NMDA receptor dependent LTP in area CA1 of the hippocampus and have been reported to show normal spatial learning in the Morris water maze (which is dependent on hippocampal function). Mice with disrupted FAK function also showed normal LTP in area CA1 of the hippocampus. Fyn tyrosine kinase deficient animals have been reported to show several neurological phenotypes, including impaired LTP induction in area CA1 of the hippocampus. This effect was found to be dependent upon the genetic background of the animal and independent of disruptions to hippocampal morphology. Short-term plasticity (paired pulse facilitation) was reduced in mice lacking fyn tyrosine kinase, an effect which was shown to be independent of age and genetic background. The mechanisms by which fyn mediates these changes were investigated through the use of experiments modulating the probability of neurotransmitter release.

Chapter I

Introduction

1.1. *Learning and memory*

The experiences that we accumulate throughout our lifetimes are maintained as memories. The ability to retain information, be it as an unconscious non-declarative process (where experience improves performance in a specific task e.g. learning a skill) or conscious declarative process (in which information is consciously known e.g. time and place of an event), allows organisms to adjust their behavior in light of previous experiences and therefore adapt to novel situations. The ability to learn is widespread, from invertebrates through to higher vertebrates and in man, a sentient being, learning and memory are essential for both survival and the development of our own unique identities.

There appears to be both a phylogenetic and anatomical difference between types of memory. Lower order organisms such as invertebrates are considered to possess non-declarative learning abilities whilst higher order organisms perform both declarative and non-declarative tasks. Particular brain regions have also been associated with different forms of memory. Clinical studies on patients suffering from amnesia and lesion studies in animals have shown the medial temporal lobe to be particularly important for declarative forms of memory and this form of memory appears to be restricted to specific brain-systems. Non-declarative memory seems to require multiple brain-systems and is often considered not to be associated with any specific brain regions.

Learning and memory are encoded by changes in the nervous system. Novel memories do not form by increasing neuronal numbers but by modulating the strength (or efficacy) of the synaptic contacts in a particular pathway. The idea that synaptic efficacy could be increased was proposed by Donald Hebb in 1949 and termed synaptic plasticity (Hebb, 1949). Synaptic plasticity has since been found to exist in several forms and in many regions of the vertebrate central nervous system (CNS). These changes in synaptic efficacy have been proposed as the cellular basis for memory.

Plastic changes are seen not only in the formation of memories and learning but also in neuronal development and neural diseases. The cellular mechanisms responsible for these types of plasticity may well have a common basis. There has been intensive study of the molecular and cellular processes that underlie the increase in synaptic efficacy, particularly in one medial temporal lobe structure, the hippocampal formation.

1.2. The hippocampal formation

Evidence that the hippocampal formation is involved in the formation and consolidation of new memories has come from many sources. Clinical studies in the late 1950s, (Scoville & Milner, 1957) reported evidence from a patient (H.M.) who had undergone surgical removal of the medial temporal lobe bilaterally to relieve severe epilepsy. Post-operatively, he suffered severe anterograde amnesia, i.e. selective amnesia for recently acquired information with sustained memory of events preceding the surgery. This indicated that there is a physiological basis for the distinction between short-term memory and long-term memory which appears to be based in the hippocampal formation (reviewed by Milner et al., 1998). Another patient, R.B., suffered a similar form of amnesia following an ischemic event during heart surgery and post-mortem analysis revealed a selective bilateral lesion of the CA1 field of the hippocampus (Zola-Morgan et al., 1986). Various considerations suggest the underlying cause of amnesia in the above patients was hippocampal damage. Patients with similar conditions have been assessed with high-resolution magnetic resonance imaging to reveal the underlying lesion and again hippocampal abnormalities appear to correlate with anterograde amnesia (Press et al., 1989).

Animal models both in invertebrates and vertebrates (especially rodents and primates) have been used to elucidate the cellular and molecular mechanisms of learning. Lesion studies in primates have been used to identify the particular brain structures required for declarative memory tasks (those memories that can be considered conscious, i.e. 'knowing that'). The hippocampal formation and

associated cortical regions, the perirhinal and parahippocampal cortices, were found to be absolute requirements for the acquisition of such tasks (Squire and Zola-Morgan, 1991).

Lesions of the hippocampal formation in rodents led to the inability of animals to determine their location in space (Olton et al., 1978). The development of the Morris water maze behavioral task provided a rigorous test paradigm for spatial learning. Morris et al. (1982, 1984) developed a water maze that tests the ability of a rodent to retain the memory of the position of an escape platform. Briefly, the animal is placed in a large, circular pool filled with opaque liquid in which a submerged escape platform is located in one of four set locations. Distal visual cues in the room are used as place markers for the animals to orientate themselves. Animals can learn the position of the escape platform through repetitive trials and video recording of the swim pathway allows analysis of the search strategy used. This is then followed by the transfer test in which the platform is removed and the time spent in each quadrant of the pool is calculated. Animals, which have successfully learnt the location of the platform, spend a significant amount of time searching the quadrant where the platform was located. Animals with lesions to the hippocampal formation however lose the ability to locate the platform, show a more random search path and spend an equivalent amount of time in each quadrant (Morris et al. 1982). Experiments in which *N*-methyl-D-aspartate (NMDA) glutamate receptor activation is blocked, using a specific antagonist aminophosphonovaleric acid (AP-5 or AP-V), also disrupted this learning ability and suggested a link between NMDA receptor activation in the hippocampus and spatial learning in the Morris water maze (Morris et al., 1986). The Morris water maze is now established as a well characterised and widely used test of hippocampal dependent learning in rodents.

The hippocampus seems to be particularly important for spatial information in rodents, and the discovery of 'place cells' (cells which only fire when the animal is in a particular location within a particular environment) in the hippocampus extends this idea (O'Keefe and Dostrovsky, 1971). Place cells are also dependent on the activation of NMDA receptors (Kentros et al., 1998). In humans, spatial awareness

in a virtual environment is also linked to hippocampal function (Maguire et al., 1998). Although spatial learning is an important function of the hippocampus, it underlies a more general role in declarative memory processing, encoding relationships between many sensory and cognitive modalities (Bunsey and Eichenbaum, 1996, Eichenbaum 1996). The hippocampal formation appears to be a structure which is deeply involved in the formation of new declarative, long-term memories and warrants further investigation into its electrophysiological, behavioral and anatomical properties (for reviews, see Alkon et al., 1991, Zola-Morgan et al., 1986, Squire et al., 1993, Squire, 1992, Nadel and Moscovitch, 1997, Thompson and Kim, 1996).

1.2.1. Anatomy of the hippocampal formation

The hippocampal formation is an elongated C shaped structure with the long (septo-temporal) axis running from the septal nuclei rostrally to the incipient temporal cortex ventrocaudally. The hippocampal formation comprises four main areas, the entorhinal cortex (medial & lateral), the dentate gyrus, the hippocampus itself (which can be divided into three sub-fields, also known as the cornu ammonis (CA) fields, CA1, CA2 & CA3) and the subicular complex (comprising of the subiculum, presubiculum & parasubiculum). The four main areas and their connections were first described by Raymon y Cajal and Lorente de No in their classic Golgi staining studies (Ramon y Cajal, 1893, Lorente de No, 1933, Lorente de No, 1934). These were later described, in greater detail, in a series of degeneration studies (Blackstad, 1956, Blackstad et al., 1970 and Raisman et al., 1965). The main areas are connected by a series of unidirectional excitatory pathways termed the tri-synaptic pathway, all of which are mediated by the transmitter L-glutamate (Watkins and Evans, 1981, Cotman et al., 1981, Fonnum, 1984).

The perforant pathway (pp) runs from the entorhinal cortex to the granule cell layer of the dentate gyrus (stratum granulosum). The dentate gyrus projects axons to the pyramidal cells of the CA3 region via the mossy fibre (mf) pathway. The Schaffer

collaterals (sch) connect the CA3 region to the pyramidal cells of the CA1 region. Hippocampal regions display several distinct layers, in the CA1 region these are: the alveus - where pyramidal cell axons run to other cortical areas such as the subiculum, the stratum oriens - a region in which the basilar dendrites of the pyramidal cells lie, the stratum pyramidale - where the cell bodies lie, and the stratum radiatum / stratum lacunosum-moleculare - where the apical dendrites connect with the Schaffer collaterals.

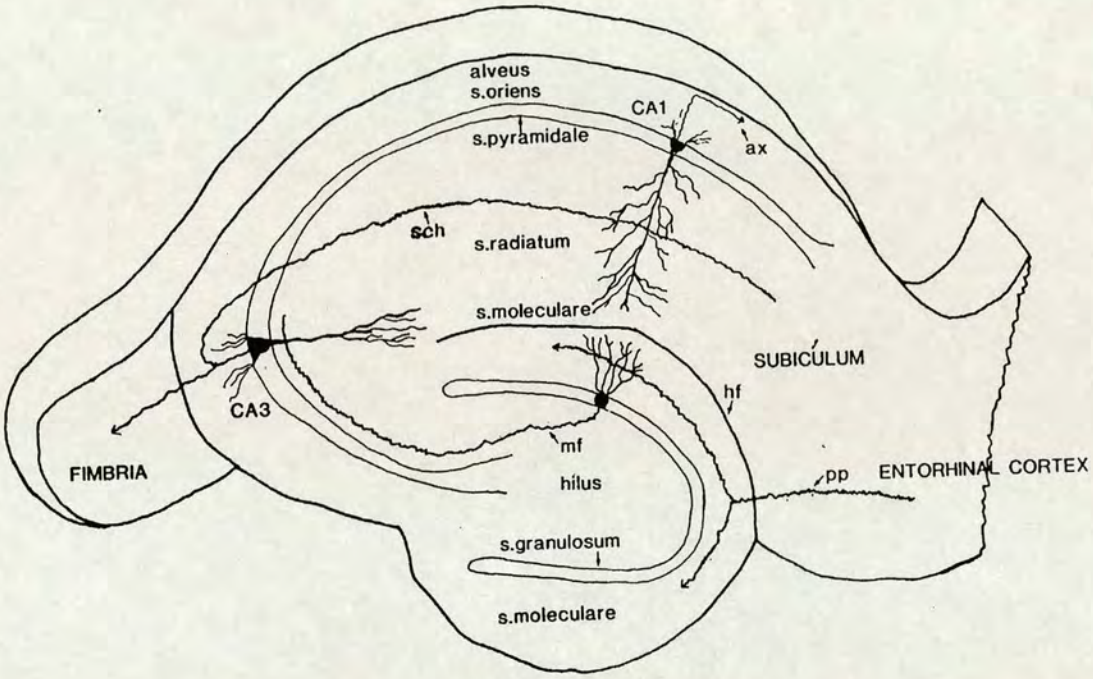


Figure 1 – Schematic diagram of the hippocampal formation
(Abbreviations: see text and *hf*- hippocampal fissure, *ax* – axons of CA1 pyramidal neurones, *hilus* – the dentate gyrus hilus)

The hippocampus contains a significant proportion of interneurones, which release the inhibitory neurotransmitter γ-aminobutyric acid (GABA). These tightly regulate the excitatory connections, and are responsible for controlling the overall level of excitability of the hippocampus. There are many different types of interneurones, including the basket cells (which contact pyramidal cells via axo-somatic synapses), chandelier cells, oriens / alveus interneurones, and lacunosum-moleculare

interneurones. These classifications are based on the cells shape, location and neurophysiological properties (Han et al., 1993).

The hippocampus receives cortical inputs from the contralateral hippocampus, associational fibres from other ipsilateral hippocampal regions, the dentate gyrus, the subiculum and the entorhinal cortex. The majority of these connections are mediated by excitatory amino acids and some co-release various peptidergic neurotransmitters. Sub-cortical inputs are also received from the septum, the hypothalamus, the raphe nuclei and the locus ceruleus. These inputs tend to have modulatory rather than direct effects on hippocampal activity, and are mediated by neurotransmitters such as GABA (septal input), serotonin (raphe nucleus), norepinephrine (locus ceruleus) and acetylcholine (ACh). These inputs can also be classified functionally, and broadly speaking, the cortical regions provide highly processed sensory information, whereas the sub-cortical areas modulate the background activity in the hippocampus.

The pathways within the hippocampus can be roughly divided into 3 groups, the lamellar circuits (the tri-synaptic pathway, Figure 1), the longitudinal pathways (many diffuse projections which run in the septo-temporal axis, which play an important function in the organisation of activity in the hippocampus, Amaral and Witter, 1989), and the local circuits (many regions project internally to interneurones and other excitatory cells). The lamellar theory suggested by Anderson et al. in 1971, described the hippocampus as a series of laminae, transversely orientated to the long axis, each of which was functionally distinct from its neighbours (i.e. each lamellae processed information within its own circuits). This lamellar structure of the tri-synaptic pathway is maintained in the *in vitro* hippocampal slice preparation (a transverse 200 - 400 μm slice) which is now commonly used to study the synaptic properties of neurotransmission in the hippocampus.

The major efferent output of the hippocampus is through the axons of the CA1 pyramidal cells to the subiculum, the entorhinal cortex, the lateral septal nucleus, the olfactory bulb, nucleus accumbens, amygdala and the hypothalamus. The CA3 region also has a dense projection to the lateral septal nucleus, from both pyramidal

and non-pyramidal cells. The hippocampal formation is a closely interconnected neural system with links to the limbic system, responsible for many autonomic and motivation functions which can influence learning. Through its outputs to the thalamus and the hypothalamus, the hippocampus can have effects on the somato-motor, autonomic and hormonal systems of the body.

1.2.2. Synaptic transmission in the hippocampal formation

Glutamate receptors are expressed in all neuronal cell types in the hippocampus and the levels of expression are some of the highest seen in the mammalian CNS (Monaghan et al., 1984). Excitatory synaptic transmission is primarily mediated by α-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid (AMPA) receptors and *N*-methyl-D-aspartate (NMDA) receptors. Inhibitory synaptic transmission is mediated by γ-aminobutyric acid (GABA) receptors. In the CA1 region Schaffer collateral stimulation produces a tri-phasic response in the post-synaptic cell, characterised by a fast excitatory post synaptic potential (EPSP), followed by two inhibitory post synaptic potentials (IPSP). The EPSP, during low frequency stimulation, is almost entirely mediated by AMPA receptors (Bekkers and Stevens, 1989).

These receptors directly gate an ion channel permeable to mainly sodium and potassium ions, and have fast kinetics. Opening of these channels, by glutamate binding, causes a sudden influx of sodium ions, and an efflux of potassium ions, depolarising the membrane potential and producing the steep rising phase of the EPSP. NMDA receptors do not play a significant role in normal low frequency transmission, due to their voltage dependent blockade by magnesium ions and their slow kinetics (Collingridge et al., 1988a and 1.3.2.b.). The IPSP is produced by GABA-ergic inhibitory interneurones. The first portion (fast phase) is mediated by $GABA_A$ receptors, which directly gate a channel permeable to chloride ions. The slow phase of the IPSP is mediated by $GABA_B$ receptors, which are indirectly linked to a potassium channel. Activation of these receptor channels in this case causes a hyperpolarisation of the membrane potential, limiting the EPSP. Inhibition plays a

significant role in modulating the synaptic responses seen in the hippocampus under both low frequency and high frequency stimulation (Davies et al., 1991, Davies and Collingridge, 1996).

1.3. Glutamate receptors

Glutamate receptor channels have been defined by their pharmacological specificity for distinct agonists. Four major groups of CNS glutamate receptors have so far been identified in this manner, the NMDA receptor, the AMPA receptor, the kainate receptor and the metabotropic family of glutamate receptors. Metabotropic receptors are linked to second messenger systems, through G-proteins and have a wide variety of functional roles. The AMPA, NMDA and kainate receptors are termed 'ionotropic receptors' collectively, as they mediate their actions through integral ion channels. The proposed stoichiometry of ionotropic glutamate receptor subunits based on their molecular sequences is a tetrameric structure, in which multiple homomeric or heteromeric subunits form the channel pore region (Rosenmund et al., 1998). This tetrameric structure is similar to the voltage activated potassium channel family and unlike other ligand-gated channels (such as the nicotinic acetylcholine receptor that is a pentamer). Biochemical and biophysical evidence suggest four ligand-binding sites on functional NMDA receptor channels also suggesting a tetrameric structure (Laube et al., 1998), although a pentameric arrangement for this receptor has been proposed (Premkumar and Auerbach, 1997).

1.3.1. AMPA receptors

AMPA receptors mediate fast excitatory actions of L-glutamate in the mammalian CNS (Mayer and Westbrook, 1987, Nicoll et al., 1990, Collingridge and Lester 1989). AMPA receptors have fast kinetics (in the order of milliseconds) and are permeable to sodium and potassium and also calcium ions depending on their subunit composition. Four receptor subunits have been cloned and are termed GluRA,

GluRB, GluRC and GluRD (Hollmann et al., 1989, Keinänen et al., 1990) or GluR1-4 (Sommer et al., 1990, Sommer et al., 1991). Each of these subunits is approximately 900 amino acids in length, has four transmembrane (TM) domains and has a large extracellular amino (N) terminal domain and an intracellular carboxyl (C) terminal domain (Seeberg, 1993, Hollmann and Heinemann, 1994). They exist in two different states, termed 'flip' and 'flop' which occur from alternative splicing of 38 residues that precede the last transmembrane domain (TM IV). These two forms show different expression profiles in the mature and developing brain. The 'flip' form of GluRA-D is expressed prenatally and continues throughout life. The 'flop' form is only seen postnatally and is often co-expressed with the 'flip' form (Sommer et al., 1990). These alternate forms have been shown to have functional consequences on the response of these channels to L-glutamate. Receptors formed *in vitro* from subunits in the 'flip' state have slower desensitization kinetics than those formed from subunits in both the 'flip' and 'flop' forms. These kinetics affect the ratio of steady state to peak components of the glutamate currents, thus channels formed solely from 'flop' forms show virtually no steady state component (Sommer et al., 1990).

Channels formed from homomeric GluRA, GluRC and GluRD subunits all show calcium ion permeability and a doubly rectifying I-V curve, *in vitro*. Homomeric GluRB channels show a linear I-V curve and low calcium permeability (Burnashev et al., 1992, Burnashev, 1996). The GluRB subunit seems to be heavily involved in the regulation of the current - voltage (I-V) relationship and divalent permeability of AMPA receptors. This unique property of the GluRB subunit comes from a single amino acid residue in the TM II region termed the Q/R site. In GluRB, this residue is arginine (R), whereas in all the other subunits this residue is glutamine (Q). However, point mutations at the Q/R site produce channels with high calcium ion permeability and linear I-V curves. This implies that the properties of the channel are controlled by more than one site, not just the Q/R locus. Naturally occurring calcium ion permeable AMPA receptor channels are seen to exist in cells that lack GluRB expression. It is possible that neurones may regulate the amount of calcium passing through AMPA receptor channels by controlling the level of GluRB

expression (Jonas and Burnashev, 1995). Different patterns of expression of GluRB subunits are seen during development. Polymerase chain reaction (PCR) analyses of embryos at day 14 have shown the presence of unedited GluRB cDNAs, whereas virtually all post-natal GluRB cDNAs are in the edited form (Sommer et al., 1991). Functionally, unedited GluRB subunits can form calcium permeable AMPA receptor channels, the edited version cannot. The role of calcium permeable AMPA receptors is unclear, but interestingly evidence suggests that native hippocampal GABA interneurones and pyramidal cells express these channels (Koh et al., 1995a, Jonas et al., 1994, Racca et al., 1996). Also, GluRB mRNA is down regulated following epileptic activity and ischemic events suggesting that calcium permeable AMPA receptors have a physiological function (Pellegrini-Giampietro et al., 1997).

The mRNA expression pattern for the receptor subunits varies widely across cortical structures. GluRB mRNA is expressed at high levels in the cerebral cortex, whilst GluRA, C and D mRNA expression levels differ among cortical layers. The hippocampus expresses high levels of mRNA for the GluRA, B and C subunits in the dentate gyrus and pyramidal cell layer. GluRD expression however is high in CA1 and in the dentate gyrus and found at much reduced levels in areas CA3-CA4 (Keinänen et al., 1990).

Recently, two molecules involved in the localisation and clustering of AMPA receptors have been identified, GRIP1 and GRIP2 (*G*lutamate *R*eceptor *I*nteracting *P*rotein, Dong et al., 1997, Dong et al., 1999). The C terminal domain of AMPA receptor subunits GluRB and GluRC bind GRIP (via a protein protein interaction domain termed a PDZ domain, Cho et al., 1992, Kistner et al., 1992). GRIP is thought to act as a scaffolding molecule for receptor clustering and synaptic localisation as well as an adapter protein linking the receptor to signalling proteins required for downstream signalling events. The PDZ motif, named after PSD-95, Dlg-A and ZO-1 (the first three PDZ containing proteins to be identified), is found in molecules seen to interact with other ion channels, such as the NMDA receptor, AMPA receptor, metabotropic glutamate receptor and the Shaker-type potassium channel. This new family of PDZ domain containing proteins (GRIPs) differs from

other PDZ containing proteins, such as PSD-95, displaying 7 PDZ repeat domains (not 3 as in the PSD-95 family) and no catalytic domain. The C terminus of the AMPA receptor subunit GluRB/C has also been shown to bind to a membrane fusion protein (NSF, Nishimune et al., 1998) and the PDZ domain of the protein kinase C (PKC)-interacting protein PICK1 (Xia et al., 1999). The presence of this structural PDZ containing protein co-localised with AMPA receptors allows the formation of a macromolecular complex, tethering important signalling and regulatory molecules close to ion influx through this receptor (Dong et al., 1999). If proteins associated with this complex have a role in the regulation of AMPA receptor properties, then any molecules found to interact with these GRIP proteins could play significant roles in synaptic plasticity involving AMPA receptors.

1.3.2. NMDA Receptors

NMDA receptor channels have two exceptional properties. First, the receptor controls a cation channel of high conductance (50 pS) that is permeable to calcium as well as sodium and potassium (Mayer & Westbrook, 1985). Second, the channel is blocked in a voltage dependent manner by magnesium at normal resting membrane potential (around -65mV, Ascher & Nowak, 1988). The channel does not conduct ions effectively when activated synaptically by L-glutamate unless there is sufficient membrane depolarisation to drive the magnesium out of the channel. These unique properties of the NMDA receptor allow it to act as a coincidence detector, as both postsynaptic membrane depolarisation and presynaptic glutamate release are required for its activation.

1.3.2.a. Calcium entry through NMDA receptors

Direct evidence that the NMDA receptor channel is a major route of calcium entry was presented by MacDermott et al. (1986) and Mayer et al. (1987). They showed that NMDA receptor agonists produce calcium entry in cells voltage clamped at -

60mV with the calcium sensitive fluorescent dye arsenazo III (i.e. in conditions excluding calcium entry through voltage gated calcium channels, VGCCs). The functional importance of this observation cannot be over estimated since calcium entry through NMDA receptor channels appears to trigger the main physiological effects of this receptor (e.g. long-term potentiation, production of arachidonic acid (AA), rhythmic activity) and is probably the main cause of cellular toxicity produced by over-activation of these receptors by both endogenous and exogenous NMDA receptor agonists. Furthermore, calcium entry produced in physiological conditions by NMDA receptor activation is likely to be highly localised to the postsynaptic density / dendritic spine, in contrast to the calcium influx through voltage gated calcium channels (due to their different localisation, Malinow et al., 1994, Gold and Bear, 1994).

The quantitative analysis of the calcium permeability of the NMDA channel is very complex. Single channel observations at various calcium concentrations show reversal potential shifts, and shifts in the current-voltage relationships (I-V) at depolarised potentials (Ascher & Nowak, 1988). This indicates that increasing the extracellular calcium concentration may affect the surface potential at the entrance to the channel. This effect may lead to an over-estimation in the relative calcium / sodium permeability, as a more negative surface potential will tend to cluster calcium ions, rather than sodium ions, around the mouth of the channel. It appears that the NMDA channel does not discriminate specifically between calcium and sodium ions, but more generally between calcium and monovalent ions.

1.3.2.b. The voltage dependent magnesium block of NMDA receptors

A 'non-competitive' antagonism was first used to describe the blockade of the glutamate response by magnesium (Ault et al., 1980). The first patch clamp analysis of glutamate responses of vertebrate central neurones was carried out by Nowak et al., in 1984. Initially they were unable to detect single channel currents above 10pS (the limit of resolution), but in the absence of magnesium, large conductances (50pS)

were seen. The re-introduction of magnesium ions at different concentrations caused the single channel current to change from relatively long openings (7ms duration) to bursts of fast events. This effect was only seen at negative membrane potentials and increased with hyperpolarisation. This gives rise to the non-linear current-voltage relationship seen in NMDA receptors in the presence of magnesium. The current-voltage relationship that these receptors display is linear in the absence of magnesium, however when magnesium is present there is little change in the outward current but a significant reduction in the inward current.

It was assumed that this effect of magnesium was due to 'channel block' (as Neher & Steinbach saw similar effects in 1978 when studying antagonists of the ACh receptor). The 'channel block' model predicts that the magnesium ion acts by plugging the channel, binding to a site located inside the transmembrane field. This idea was modified to the 'open channel block' hypothesis (where magnesium can only enter and leave when the channel is open), and used to evaluate the rate constants for magnesium binding (Nowak et al., 1984). Ascher & Nowak (1988) showed that the duration of short openings is dependent on magnesium concentration, whilst the duration of short closures is not. Depolarisation increases the duration of short openings, and decreases the duration of short closures. These results do not fit with a simple 'open channel block' model, and it has become clear that the NMDA receptor channel has very complex gating properties showing multiple sub-conductance states and opening and closing kinetics. The possibility of a further intracellular voltage dependent magnesium block has also been suggested (Nowak et al., 1984, Li-Smerin and Johnson, 1996a, 1996b).

1.3.2.c. Glycine as a co-agonist of NMDA receptors

Glycine is an inhibitory transmitter in the vertebrate CNS, and has been shown to play a crucial role in the activation of NMDA receptors. Johnson and Ascher showed that altering the extracellular concentration of glycine significantly affected the currents of NMDA receptor channels. Decreasing the concentration of glycine led to a large reduction in the current evoked by NMDA receptor agonists. The

glycine-binding site exists on the extracellular surface of the NR1 receptor subunit and is distinct from the glutamate-binding site on the NR2 subunit, and for significant current flow to occur, glycine must be bound. *In vivo*, it is proposed that there is a saturating concentration of glycine (Johnson and Ascher, 1987), however recent evidence using inhibitors of both glycine and the glycine transporter type 1 (GLYT1) has suggested that glycine binding sites are not always saturated (Bergeron, 1998). Experiments in cortical slices have shown that a tonic regulation of NMDA receptor mediated neuronal excitation by glycine occurs (Thomson et al., 1989). Experiments in glycine free solutions have suggested that it may be impossible to open NMDA channels, without the glycine site bound (Kleckner and Dingledine, 1988). NMDA receptor currents are seen to desensitise during prolonged NMDA application (Mayer and Westbrook, 1985) and glycine enhancement of NMDA receptor currents occurs in part through an acceleration of recovery from desensitisation (Mayer et al., 1989).

1.3.2.d. Molecular biology of NMDA receptors

NMDA receptors are unique among ligand gated ion channels, due to their voltage dependent magnesium block. Six receptor subunits: NR1A; NR1B; NR2A; NR2B; NR2C and NR2D, have been cloned (Moriyoshi et al., 1991, Durand et al., 1992). A novel NMDA receptor subunit NR3 has recently been reported (Das et al., 1998), the role of which is not yet clear. The NR1 subunit is considered as the principal constituent of the NMDA receptor channel, and can form homomeric channels that display many features of native NMDA receptors (Moriyoshi et al., 1991). These homomeric channels however show low current amplitudes when compared to channels formed from both NR1 and NR2 subunits (some 100 times larger, Monyer et al., 1992) indicating that *in vivo* NMDA receptors are heteromeric. The different NR2 subunits modulate the functional properties of the channel, including the strength of the magnesium blockade, glycine sensitivity, kinetics of channel gating and single channel conductance. The permeation and blocking properties of divalent ions is determined by an asparagine residue in TM II of the NR1 and NR2 subunits

(Molinoff et al., 1994, Monyer et al., 1994, Williams et al., 1994, Lynch et al., 1995). Native NMDA receptors are thought to contain two NR1 and two NR2 subunits, forming a tetrameric structure (Behe et al., 1995, Laube et al., 1998). NR2 subunits show distinct regional and developmental distributions (Sheng et al., 1994, Monyer et al., 1994). In the adult brain NR2A is expressed ubiquitously, NR2B is mainly localised to the forebrain and NR2C in the cerebellum, whereas NR2D subunits are found only in very young animals (Monyer et al., 1994).

The NMDA receptor forms a macromolecular complex at the postsynaptic density, by binding to scaffolding protein of the membrane associated guanylate kinases (MAGUKs, e.g. PSD-95) family through the NR2 subunits (Kornau et al., 1995, Kim et al., 1996, Muller et al., 1996, Lau et al., 1996, Niethammer et al., 1996). Postsynaptic density protein 95kD (PSD-95) was the first family member to be identified (Cho et al., 1992, Kistner et al., 1993) and all family members (including chapsin-110 / PSD-93, SAP97 and SAP102) have three PDZ domains, an SH3 (src homology 3) domain and a guanylate kinase-like domain (GK). PSD-95 is expressed primarily in the brain. Its expression increases rapidly at post-natal days 8-18, during the time that long-term depression (LTD) diminishes and long-term potentiation (LTP) emerges. This suggests a role for this protein in the regulation of NMDA receptor dependent synaptic plasticity. The first two PDZ domains of PSD-95 has been shown to bind to several different NMDA receptor subunits, NR2A, NR2B, NR2D, the shaker type potassium channel Kv1.4 and the inward rectifier potassium channels Kir2.3 and Kir 4.1 (Cohen et al., 1996, Horio et al., 1997).

PSD-95 acts as a clustering and localisation molecule for these receptors by binding to cytoskeleton proteins such as actin. The other binding domains of this molecule allow binding to a variety of proteins, such as the non-receptor tyrosine kinases src and fyn, the plasma membrane calcium ATPase, neuronal nitric oxide synthase, the cell adhesion molecule neuroligin and the ras pathway activating protein synGAP (Kim et al., 1998, Brenman et al., 1996, Irie et al., 1997, Chen et al., 1998). Mice expressing a truncated form of PSD-95 (only the first two PDZ domains) display normal gross hippocampal structure, normal ultra-structure of synapses and NR1

subunit localisation. Basal synaptic transmission appears normal in these mice, however paired pulse facilitation, a form of short-term plasticity, was increased. LTP in these mice is dramatically increased and even LTD protocols (that produced depression in wild type littermates) induced LTP in the PDS-95 mutant mice. It appears that PSD-95 and the associated proteins act as a negative regulator of LTP. Spatial learning in the Morris water maze is also severely affected in the PSD-95 mutant mouse (Migaud et al., 1998). The recent discovery of scaffolding molecules for a number of ion channel groups is an important step in our understanding of how the ion channels can mediate the intracellular signalling cascades required for phenomena such as synaptic plasticity.

1.3.3. Metabotropic glutamate receptors (mGluRs)

mGluRs are distinct from ionotropic glutamate receptors as they are linked to guanine nucleotide binding proteins (G-proteins). mGluRs were first discovered in cultured striatal neurones (Sladeczek et al., 1985). It was seen that L-glutamate activates phospholipase C (PLC) in preparations where ionotropic channels had been selectively blocked. These results indicated that L-glutamate activated both receptors linked to ligand gated ion channels and G-protein coupled receptors. The existence of a novel receptor was confirmed by using the Xenopus oocyte model, which endogenously expresses these receptors (Sugiyama et al., 1987). To date, eight genes coding mGluRs have been cloned, several of which produce alternatively spliced mRNAs (reviewed in Pin & Duvoisin, 1985). These have been termed mGluR1-8.

All mGluRs have a general topography of an extracellular amino terminal and 7 hydrophobic membrane spanning units. The carboxy terminal varies in length and is presumably intracellular. Twenty one cystine residues are also conserved throughout the mGluR family, and there is homology in the proposed ligand binding domains and the G-protein binding domains. Recently this group of receptors has also been shown to bind to a scaffolding protein termed homer, which is unusual as it contains

only one PDZ binding domain (Brakeman et al., 1997). Interestingly the expression of homer is regulated as an immediate early gene and is dynamically responsive to synaptic activity. As is the case for PSD-95 / NMDA receptors and GRIP / AMPA receptors, this molecule is thought to both localise receptors and act as a signal transduction molecule.

1.3.4. Kainate receptors

Kainate receptors are widely distributed in the mammalian CNS. The receptor family compromises of subunits: GluR5, GluR6, GluR7, KA1 and KA2. Active receptor complexes can form from various homomeric and heteromeric configurations, excluding homomeric GluR7, KA1 and KA2 configurations. GluR5 and GluR6 subunits occur in two forms with respect to the amino acid residue occupying the Q/R site in TM II. RNA editing of the GluR6 subunit (in the TM I region) leads to seven different edited forms and one genomically encoded form. Sixty five percent of receptor complexes in the adult rat brain contain the GluR6 subunit in its fully edited form (i.e. both in the TM I and TM II regions), whilst genomically encoded forms only account for about ten percent (Sommer et al., 1991).

Kainate receptor channels are permeable to sodium and potassium ions (and calcium ions in channels containing GluR6 subunits), and are often considered to be similar to AMPA receptors. However, the molecular diversity of this family and the expression pattern in certain regions of the CNS indicates a different functional role to AMPA receptors. Application of kainic acid activates a large non-desensitising current in AMPA receptor channels, which may overshadow the rapidly desensitising currents of kainate receptors. The functional significance of kainate receptor type glutamate channels is, at the present time, poorly understood although these receptors have been implicated in epileptogenesis and cell death (Meldrum and Garthwaite, 1990).

In the hippocampus, expression of kainate receptor subunits is high in the CA3 regions, and lower in the CA2 region. In the CA3 region a slow frequency dependent postsynaptic current, mediated by kainate receptors, has recently been identified in the mossy fibre pathway, using specific AMPA receptor antagonists. This current can greatly augment the excitatory drive of CA3 pyramidal cells, under high frequency stimulation, suggesting a role in synaptic plasticity (Castillo et al., 1997, Vignes and Collingridge, 1997). The lack of specific kainate receptor antagonists and agonists has impaired the understanding of this receptor subtype. A novel agonist (ATPA) and antagonist (LY294486) against the kainate receptor GluR5 subunit has been recently identified and its actions examined in hippocampal slices. Application of ATPA reversibly depressed monosynaptically activated inhibitory postsynaptic potentials (the same action has been reported for kainate, Rodriguez-Moreno et al., 1997) and was reversed by the antagonist LY294486 (Clarke et al., 1997). As such these effects on inhibitory synaptic transmission may lead to a better understanding of how kainate receptors are involved in epileptogenesis.

1.4. GABA receptors

Inhibition of synaptic transmission in the central nervous system is mediated primarily by the amino acid γ-aminobutyric acid (GABA, Sivilotti and Nistri, 1991). There are distinct neuronal populations of GABA mediated inhibitory interneurones found throughout the hippocampus and cortex. These neurones have different architectures, localisation and physiological properties, and inhibit excitatory transmission in several ways. At least five different types of local circuit inhibitory neurones may be discriminated in the dentate gyrus (Han et al., 1993). Three of these types pinpoint different segments of the granule cells dendritic arbour and may thus be able to act locally and specifically with particular excitatory synaptic inputs (Halasy and Somogyi, 1993). In contrast, the basket cell (identified by Ramon Y Cajal) targets the cell soma and dendritic stem and may therefore govern the somatic integration of inputs. The terminals of the axo-axonal cell are closest to the initiation

site of the action potential and may thus control the output and firing rate of neurones. These inhibitory interneurones are small in number but have a very extended architecture, individual cells can form hundreds of inhibitory synapses on pyramidal cells, often making several contacts onto the same cell (Buhl et al., 1994, Cobb et al., 1995, Li et al., 1992, Sik et al., 1995). A form of LTD has been identified at interneurones (iLTD) in response to high frequency stimulation, further indicating a role for inhibition in the control of synaptic plasticity and transmission (McMahon and Kauer, 1997).

The GABA family of receptors includes a ligand gated ion channel receptor, $GABA_A$, which mediate the fast inhibitory component. There are currently 16 known $GABA_A$ receptor subunits that form a pentameric heteromeric chloride channel. The subunits are termed α1-6, β1-3, γ1-3, δ and ρ1-2 (Schofield et al., 1987, Levitan et al., 1988, Pritchett et al., 1989, Pritchett and Seeberg, 1990, Shivers et al., 1989, Ymer et al., 1989, 1990, Lüddens et al., 1990, Wisden et al., 1991, Wilson-Shaw et al., 1991, Cutting et al., 1991, Hadingham et al., 1993). Due to the large number of subunits the possible permutations of receptor assemblies are over half a million, however some subunit compositions are preferred during assembly in a native environment (Nayeem et al., 1994, Backus et al., 1993, Angelotti et al., 1993). The α, β and δ subunits are all required to form a functional chloride channel. $GABA_A$ receptor activation leads to hyperpolarisation, little or no change in membrane potential or to depolarisation depending on the chloride reversal potential and the resting membrane potential of the neuron. No matter which direction the membrane potential deviates during an inhibitory postsynaptic potential (IPSP) activation leads to large increases in membrane conductance and a strong shunting of excitatory currents (Mody et al., 1994, Staley and Mody, 1992). Another form of GABA receptor has been proposed ($GABA_C$, Johnston, 1996) although this now appears to be a subclass of $GABA_A$ receptors based on its molecular sequence (Kaupmann et al., 1997).

The second class of GABA receptors is the $GABA_B$ receptor. $GABA_B$ receptor cloning has proved elusive although two isoforms designated $GABA_BR1$ and

$GABA_BR2$ has recently been cloned (Kaupmann et al., 1997, Bowery and Brown, 1997). This channel is similar in structure to other G-protein linked receptors, has 7 transmembrane domains and modulates synaptic transmission through intracellular effector systems. Activation of the receptor stimulates the G-protein (G_i) which inhibits adenylate cyclase and opens a potassium channel. Another G-protein (G_o) retards the opening of a calcium channel (Clapham and Neer, 1993, Campbell et al., 1995, Ikeda et al., 1996, Herlitze et al., 1996). $GABA_B$ receptors are found both presynaptically and postsynaptically and mediate different effects. Presynaptic $GABA_B$ auto-receptors, by activation of a potassium conductance and inhibition of calcium conductances, inhibit release of GABA, reducing the overall level of inhibition (Khazipov et al., 1995). This inhibition is frequency dependent and leads to the inhibition of GABA release during high frequency stimulation and regulates the induction of LTP (Davies et al., 1991, Davies and Collingridge, 1996, Wigstrom and Gustafsson, 1985). Postsynaptically, $GABA_B$ receptors mediate a slow inhibitory potassium conductance that in conjunction with the fast $GABA_A$ mediated chloride conductance opposes excitatory postsynaptic potentials (Algar and Nicoll, 1982, Dutar and Nicoll, 1988, Otis et al., 1993).

GABA receptors also interact with the scaffolding protein GRIP1, perhaps localising them to the same sites as AMPA receptors (Dong et al., 1999). $GABA_A$ receptors are modulated by tyrosine phosphorylation. Phosphorylation of tyrosine residues within the γ2L and β1 subunits enhanced whole cell GABA mediated currents when co-expressed with src (Moss et al., 1995). Also GABA mediated currents are increased upon the intracellular injection of src to cultured CNS neurones (Wan et al., 1997, Herron and Grant, 1997).

1.5. Short-term potentiation

Synaptic transmission can be modulated in many ways (plasticity), by alterations in the presynaptic release machinery, the postsynaptic sensitivity, the integration of excitatory and inhibitory inputs and even by morphological alterations. There are forms of short and long-term plasticity seen at many synapses in the peripheral and central nervous systems. Of particular note is the short-term phenomenon of paired pulse facilitation (PPF) which is seen in area CA1 of the hippocampus. If synapses are stimulated twice in rapid succession (< 500ms between impulses) there is facilitation of the second (test) response compared to the first (conditioning) response.

The residual calcium hypothesis, proposed by Katz and Miledi (1968), is commonly used to explain PPF. The first action potential invades the presynaptic terminal, calcium influx occurs, and neurotransmitter is released, producing a postsynaptic response. The calcium elevation in the presynaptic terminal declines in a matter of seconds (dependent on the rate of calcium efflux by active membrane pumps and the sequestering of calcium by intracellular compartments). Thus, if a second action potential invades the terminal before the intracellular calcium level has reached baseline, this 'residual calcium' will contribute to neurotransmitter release on the second pulse, enhancing the second response. The relationship between intracellular calcium levels and neurotransmitter release is non-linear, and is approximately a fourth or fifth power relationship, thus even though the small level of residual calcium is not capable of promoting transmitter release itself, it can contribute significantly to subsequent release. At the neuromuscular junction (NMJ), superimposed upon this facilitation under conditions of high transmitter release is a depression of the test response, which usually results from either a depletion of the releasable transmitter or a decrease in the probability of its release (del Castillo and Katz, 1954, Katz and Miledi, 1968). There are a large number of neurotransmitter vesicles available for release in response to an action potential, but each responds with a relatively low probability that is independent of the release of any other vesicle (del Castillo and Katz, 1954, Fatt and Katz, 1952). However when two

stimuli are applied close together, the amplitude of the test response is reduced when compared to the conditioning response (Betz, 1970), a phenomenon known as paired pulse depression. If the same experimental protocol is performed in conditions where the release probability has been reduced, such as increasing the divalent cation ratio (Mg^{2+} / Ca^{2+}), the depression can be converted into a facilitation (del Castillo and Katz, 1954, Katz and Miledi, 1968). This paired-pulse depression at the neuromuscular junction is not accompanied by a decrease in the sensitivity of the endplate to acetylcholine and therefore probably results from a decrease in transmitter release. At central synapses it has been demonstrated that paired-pulse depression results from a decrease in release probability, rather than a decreased postsynaptic receptor sensitivity (Debanne et al., 1996, Turner et al., 1997). PPF can be used as an approach to the study of presynaptic receptor plasticity and function that does not rely upon statistical models of transmitter release (such as quantal analysis, see below). However, false results can be seen with this technique. The response to the conditioning impulse might cause receptor desensitisation and thus a diminished response to the test impulse, hence a decrease in the amplitude ratio (the normal means of expressing PPF).

1.6. Introduction to long-term potentiation

The hypothesis that memory storage must be found among synapses which support activity dependent changes in synaptic efficacy was proposed by Donald Hebb (Hebb, 1949). The first 'Hebbian' synapses to be identified in the mammalian brain were the excitatory connections made by the perforant pathway fibres onto granule cells of the dentate gyrus. Brief trains of high frequency stimulation to monosynaptic excitatory pathways in the hippocampus cause an abrupt and sustained increase in the efficacy of synaptic transmission. This effect was first described in detail in 1973, and is termed long-term potentiation (LTP, Bliss & Lømo 1973, Bliss & Gardener-Medwin 1973). The profile of potentiation seen after an LTP inducing stimulus also includes post-tetanic potentiation (PTP) and short term potentiation

(STP) followed by LTP. The cellular and molecular processes that mediate LTP are thought to be different from both STP and PTP.

PTP is seen immediately after tetanus. It is thought to be the result of the action of calcium ions that flow into the presynaptic terminal when repetitive stimulation occurs. The large increase in calcium is thought to overload buffering mechanisms so that the resting concentration of calcium rises to the point where more synaptic vesicles will be released on the subsequent arrival of an action potential. This enhancement of presynaptic vesicle release parallels the decay of the presynaptic calcium levels and can last for many minutes. PTP can be induced by relatively weak tetani that will not induce LTP and does not require the activation of NMDA receptors.

STP is a form of potentiation that outlasts the decay of the calcium transient in the presynaptic terminal. It is dependent on the activation of the NMDAR and resultant calcium influx into the postsynaptic cell. STP can be distinguished from LTP pharmacologically as the use of kinase inhibitors does not block its formation and the application of NMDA produces only STP (Kauer et al., 1988), which lasts for approximately 30 minutes before decaying back to baseline levels. The molecular basis of STP is unclear as blockade of elements known to be required for LTP does not affect the expression of STP (Kauer et al., 1988, Malenka, 1991).

LTP has been found in all excitatory pathways in the hippocampus as well as in several other cortical regions, and there is growing evidence that it underlies at least certain forms of memory. Three distinct forms of LTP exist in the hippocampus: NMDA receptor dependent LTP seen at the Schaffer collateral / CA1 synapse and in the dentate gyrus, NMDA receptor independent LTP seen in the mossy fibre pathway and a form which is dependent on voltage gated calcium channels (VGCCs, Grover and Teyler, 1990). NMDA receptor dependent LTP has now become the standard model for activity dependent synaptic plasticity in the mammalian CNS. Its known properties, induction and suggested mechanisms of signal transduction are reported below.

1.6.1. Protocols of long-term potentiation induction

LTP can be induced in a number of ways, most conveniently by delivering high frequency tetanus to the pathway of interest. Typically, an induction protocol will consist of a train of 50-100 stimuli at 100Hz. Two other stimulation protocols are particularly relevant, as they mimic the firing patterns observed in the hippocampus during learning (Otto et al., 1991). 'Theta - burst stimulation' is several bursts of 4 shocks at 100Hz, with an interburst interval of 200ms (Larson et al., 1986). 'Primed burst stimulation' consists of a single priming stimulus, a 200ms interval, then a single burst of 4 shocks at 100Hz (Rose & Dunwiddie, 1986). Low frequency (1Hz) stimulation can also induce LTP, if paired with depolarising pulses delivered through an intracellular electrode (Kelso et al., 1986). Chemical stimulation (such as the application of calcium, arachidonic acid, carbachol and ACPD) of hippocampal synapses also induces a form of LTP, but these protocols are unaffected by NMDA antagonists suggesting that these forms of induction are acting on signal transduction pathways downstream of NMDA receptors. The VGCC form of LTP is only visible in conditions of NMDA receptor blockade and very high frequency stimulation of four 0.5s 200Hz tetani separated by 5 seconds (Grover and Teyler, 1990).

1.6.2. NMDA receptor dependent long-term potentiation

The induction of LTP produced by tetanic stimulation can be explained by the properties of the NMDA receptor and the role of synaptic inhibition. NMDA receptors exhibit a voltage dependent magnesium block. At resting membrane potentials, this blockade is very strong and only at highly depolarised membrane potentials does ion flow occur. Tetanic stimulation of hippocampal neurones causes the summation of AMPA receptor mediated responses, leading to a sustained period of depolarisation and a prolonged release of L-glutamate. In these conditions the magnesium block of the NMDA receptor channel is relieved and glutamate is available to activate channel opening (Collingridge et al., 1988b). The slow time course and voltage dependence of the NMDA receptor mediated conductance makes

it particularly susceptible to synaptic inhibition. GABA-ergic inhibitory interneurones, show frequency dependent depression of GABA release. $GABA_B$ autoreceptors exist on the presynaptic terminal and bring about a reduction in GABA release. This effect takes about 10 ms to fully develop and can last for several seconds (Davies et al., 1991). During tetani, GABA release is depressed, facilitating depolarisation of the postsynaptic spine and NMDA receptor / channel activation.

1.6.3. Long-term depression

An effective synaptic mechanism for encoding information would require a synapse to be able to enhance its activity (LTP), but would also require a mechanism of down regulating activity. Long-term depression (LTD) has been shown to exist in the hippocampus (Dudek and Bear, 1992) and appears to be more prevalent in immature animals. It can be produced by periods of low frequency (1Hz) stimulation and can be completely reversed by LTP stimuli. Homosynaptic CA1 LTD can be saturated, is input specific, requires the activation of NMDA receptors and elevates the level of intracellular calcium (Mulkey & Malenka, 1992). This form of LTD appears to be mediated by calcineurin dephosphorylating phosphatase inhibitor 1, which in turn increases protein phosphatase 1 activity contributing to LTD (Mulkey et al., 1994). Two distinct forms of LTD have been reported in CA1 pyramidal cells, one of which is dependent on NMDA receptors and requires protein phosphatase activity and one which is dependent on metabotropic glutamate receptors (Group 1), protein kinase C and T type voltage gated calcium channels (Oilet et al., 1997). Mice lacking the metabotropic glutamate receptor 2 subunit (which is expressed presynaptically in the mossy fibre-CA3 pathway) show normal LTP, basal transmission and paired pulse facilitation but display practically no LTD (Yokoi et al., 1996). It is postulated that the magnitude and duration of the Ca^{2+} signal in the postsynaptic cell may determine the whether LTP or LTD is produced (Lisman, 1985, Lisman and Goldring, 1988). Lower levels of calcium influx are thought to activate the protein phosphatases (due to higher calcium binding affinity) and higher levels of calcium influx activate the protein kinases (Staubli and Chun, 1996, Lisman, 1989).

1.6.4. The role of calcium in long-term potentiation

NMDA receptor activation opens a channel highly permeable to calcium (Ascher & Nowak 1988, Jahr & Stevens 1987). It is this calcium signal that is thought to be the trigger for the induction of LTP. Early studies showed that intracellular injection of calcium chelators blocked the induction of LTP (Lynch et al., 1983, Malenka et al., 1988). Tetanic stimulation has been shown, using calcium imaging techniques, to elevate calcium within spines for periods of up to several seconds and produce calcium gradients from spines to dendrites which can last for several minutes (Muller and Connor, 1991, Regehr et al., 1989). However, LTP induction does not require calcium transients which are maintained for this time period and can still be induced when calcium transients are limited to 3 seconds (Malenka et al., 1992). Dendritic spines are thought to spatially restrict the diffusion of calcium therefore local intracellular concentrations around the postsynaptic site will be high (Guthrie et al., 1991). Calcium induced calcium release from intracellular stores may also play an important role in LTP induction (Harvey and Collingridge, 1991). Metabotropic glutamate receptor activation generates inositol 1,4,5-triphosphate (IP_3) which brings about calcium release from intracellular stores and this can even induce LTP in the presence of NMDA receptor antagonists (Bortolotto and Collingridge, 1993). Elevating calcium levels artificially produces a form of potentiation, though this may not be equivalent to the potentiation seen in LTP. This may be due to the fact that gross changes in intracellular calcium levels is an inappropriate signal and that only restricted calcium elevation in spines can trigger the induction of LTP. Very high frequency stimulation in the presence of NMDA antagonists has also been shown to produce a slowly developing potentiation that is blocked by L-type calcium channel antagonists (Grover & Teyler, 1990). It is also unclear if this potentiation is equivalent to tetanically induced LTP.

1.6.5. Locus of expression of long-term potentiation

The question as to whether the changes seen in LTP are due to presynaptic or postsynaptic modifications remains unresolved. In reality, it is likely to be a combination of the two. A presynaptic model would require an overall increase in either the amount, or the probability, of transmitter release. This would require a retrograde messenger signal from the postsynaptic terminal in response to calcium influx through NMDA receptors. A proposed candidate is arachidonic acid (AA). It is released extracellularly in culture upon NMDA receptor activation and LTP induction increases the rate of AA efflux (Dumuis, 1988). Inhibitors of phospholipase A_2 (which liberates AA from phospholipids), block LTP and transient application of AA causes a slow onset potentiation (Williams and Bliss, 1989, Okada et al., 1989, Barbour et al., 1989, Williams et al., 1989). Another candidate is nitric oxide (NO). NO is also released upon NMDA receptor activation (Garthwaite et al., 1988) and inhibitors of nitric oxide synthase (the enzyme which produces NO) may block LTP (Williams et al., 1993). NMDA receptor mediated transmission can be modulated by the release of caged NO, although this is not sufficient to induce LTP (Murphy et al., 1994). The fundamental problem with both of these molecules is that they have a slow time course and potentiation is seen immediately after tetanus. Inhibitors of these compounds do not affect STP.

Other evidence does exist implicating a presynaptic locus for LTP induction. The amount of radiolabelled L-glutamate overflow in perfusates from hippocampal slices increased after induction of LTP, suggesting that more transmitter is released (Dolphin et al., 1982, Feasey et al., 1986). Calcium levels are raised in synaptosomes 45 minutes after LTP induction and there are changes in IP_3 levels (Lynch and Voss, 1991, Malenka et al., 1989). Presynaptic mGluRs may well be coupled to some stage of transmitter release, through arachidonic acid and cAMP pathways enhancing presynaptic transmitter release (Aramori and Nakanishi, 1992).

The application of quantal analysis to glutaminergic synapses have provided contradictory results as to the locus of expression. The extension of quantal analysis

techniques derived at the neuromuscular junction to CNS transmission is technically more difficult. This is due to several complications in the application of quantal recordings to CNS transmission. The size of the quantal events is significantly smaller than at the neuromuscular junction and higher level of background noise means that resolving single quanta can be difficult. Ideally, quantal analysis should be performed on two monosynaptically connected cells and this is possible in *in vitro* brain slice preparations, however the rate of finding pairs of connected cells is low and thus statistical analysis becomes less valid. Multiple afferent inputs into pyramidal cells also means that monosynaptic recordings are extremely difficult to obtain (even with the use of minimal stimulation). It appears that unlike the neuromuscular junction, the quantisation of CNS synapses is probably determined by the availability of postsynaptic receptors (Larkman et al., 1991).

Quantal studies have argued for a presynaptic locus, seeing changes in p (the probability of release) after LTP induction (Bekkers and Stevens, 1990), changes in the coefficient of variance ($1/CV^2$, a measure of synaptic variability) associated with presynaptic manipulations but not postsynaptic modifications (Malinow and Tsien, 1990, Malinow, 1991) and a decrease in the number of failures of transmission (interpreted as an increase in p, Malinow and Tsien, 1990). However, a small change in quantal size (indicating a postsynaptic modification) was also noted in one of these studies (Malinow, 1991). Kullmann and Nicoll demonstrated increase in the quantal content (presynaptic), the quantal amplitude (equivalent to quantal size, postsynaptic) or both after LTP induction using alternative analysis methods to the classical binomial statistics used in quantal analysis at the neuromuscular junction (Kullmann and Nicoll, 1992). A novel technique of labelling the intra-luminal domain of the synaptic vesicle protein synaptotagmin using a specific antibody demonstrated that the level of vesicle recycling was significantly enhanced during glutamate induced LTP, again arguing for a presynaptic locus (Malgaroli et al., 1995). Studies of the changes in paired pulse facilitation (PPF) with LTP have also been used to imply a presynaptic locus. However, contradictory evidence has been found with PPF being either increased or decreased following LTP induction (Schulz et al., 1994, Schulz et al., 1997, Kleschevnikov et al., 1997, Kuhnt and Voronin, 1994).

Evidence for a postsynaptic locus (suggesting a change in the quantal amplitude) has been presented by several groups, although a definite distinction between a pre or postsynaptic site is still to be resolved (Manabe et al., 1993, Wu and Saggau, 1994, Foster and McNaugton, 1991). A recent demonstration using a non-quantal method argues for the postsynaptic locus of expression (Hjelmstad et al., 1997). Postsynaptically, the simplest scheme for enhancement of synaptic transmission is through modulation of the number and / or properties of the ion channels involved in mediating the response. Evidence for activation of protein kinases raises the possibility that they may directly phosphorylate ion channels, modifying their ionic conductance. AMPA receptor sensitivity is gradually seen to increase after LTP induction and can be blocked by the protein kinase inhibitor K-252b (Reymann et al., 1990). The PKA catalytic subunit directly increases AMPA receptor mediated currents (Greengard et al., 1991, Wang et al., 1991). AMPA receptors have been shown to exist in the 'flip' and 'flop' forms which show different conductances, LTP may change the ratio of these states within the postsynaptic site although there is no experimental evidence to support this idea to date (Sommer et al., 1990). There is the possibility that LTP may alter the subunit expression within the cell altering the response of existing receptors, or by forming new receptors modulate the sensitivity of the postsynaptic site.

The NMDA receptor mediated component has been reported to show potentiation after LTP induction (Asztely et al., 1992, Bashir et al., 1991, Tsien and Malinow, 1990) but this evidence has been challenged (Kauer et al., 1988, Muller and Lynch, 1988, Kullmann, 1994). The confusing array of data implicating both the presynaptic and postsynaptic loci as the site of expression of LTP indicates that there is probably a contribution of both sites, perhaps in a temporally distinct manner to LTP (Davies et al., 1989).

1.6.5.a. Silent synapses

Recent studies have shown that a proportion of the excitatory synapses in the CA1 region express non-functional AMPA receptors and functional NMDA receptors. These cells show no detectable excitatory postsynaptic current (EPSC) at negative membrane potentials, yet show currents at positive potentials. These currents can be blocked by D-AP5, an NMDA receptor antagonist. When these cells are subjected to an LTP induction protocol, EPSCs become apparent which are mediated by AMPA receptors (Kullmann, 1994, Issac et al., 1995). This conversion of non-functional receptors to functional receptors can also be blocked by D-AP5, showing that the conversion is an NMDA dependent process. This idea strongly supports a simple postsynaptic locus for LTP, where groups of AMPA receptors can alternate between functional and silent forms, in an NMDA receptor dependent manner (Liao et al., 1995). This theory does not fit with all of the available data however. LTP of the NMDA mediated component would not accounted for by this hypothesis, but has been demonstrated. Both Stevens and Wang (1994) and Bolshakov and Siegelbaum (1995) demonstrated a single quantal peak of non-failures EPSCs, which was not altered after LTP induction, but saw a decrease in the failure rate following LTP. These data argues that no new AMPA receptors clusters are integrated after LTP induction. However, this form of enhancement of synaptic efficacy may be important for development of synaptic integration as synapses which only contains functional NMDARs are readily seen in immature animals (Durand et al., 1996, Wu et al., 1996, Issac et al., 1997).

Regardless of the locus of expression of LTP the influx of calcium through NMDA receptors postsynaptically is generally accepted as being the trigger for LTP induction in the hippocampal synaptic environment. The signalling cascades initiated by this influx are detailed below.

1.6.6. Signal transduction of the NMDA receptor mediated calcium influx

Several calcium sensitive enzymes have been proposed to play a part in converting the induction signal into persistent changes in synaptic efficacy. Interest has focused on phosphorylation cascades and in particular the role of protein kinases and protein phosphatases. A major mechanism by which the activity of proteins can be regulated is phosphorylation. Kinases (responsible for phosphorylation) and phosphatases (responsible for de-phosphorylation) play a major role in all signalling pathways, including those underlying LTP. The involvement of both serine / threonine kinases, tyrosine kinases and protein phosphatases in synaptic plasticity has been demonstrated.

The first kinase to be implicated in LTP was the calcium / phospholipase-dependent protein serine / threonine kinase (PKC). Inhibitors of this enzyme or genetic ablation have been reported to block LTP induction (Malinow et al., 1989, Abeliovich et al., 1993). PKC activation is not sufficient to induce LTP, but it is a necessary factor and may be involved in converting short-term potentiation (STP) to LTP (Malinow et al., 1988, Malenka et al., 1987, Malenka et al., 1986). PKC mediates plastic changes through its phosphorylation and modulation of AMPA receptors (Roche et al., 1996) and NMDA receptors (Ben-Ari et al., 1992, Chen and Huang, 1992, Durand et al., 1992, Tingley et al., 1993, Tingley et al., 1997). PKC has also been shown to phosphorylate GAP-43, a presynaptic neuron specific protein that may be involved in presynaptic plasticity (Gianotti et al., 1992, Ramakers et al., 1995).

Inhibitor studies on the serine / threonine calcium / calmodulin dependent protein kinase (CaMKII) indicate a role for this kinase in the induction but not maintenance of LTP (Malenka et al., 1989, Malinow et al., 1989, Otmakhov et al., 1997). Knockout of the gene encoding αCaMKII (a form enriched in the postsynaptic density) severely impairs LTP induction and spatial learning (Silva et al., 1992). CaMKII is a major component of the postsynaptic density (Kennedy et al., 1983) and can phosphorylate a number of synaptic proteins including itself. Autophosphorylation leads to a change in the enzyme properties such that it is no

longer dependent on calcium to maintain its activity (Miller and Kennedy, 1986, Fong et al., 1989, Ohsako et al., 1991). This property, which means that the kinase activity can continue beyond the duration of the activating calcium signal has lead to the proposal that kinase underlies at least the early stages of LTP and has been termed a 'memory molecule'. LTP induction increases the phosphorylation levels of CaMKII and mice expressing a mutant form of CaMKII which cannot become calcium independent cannot produce LTP (Fukunaga et al., 1993, Giese et al., 1998). Potential targets of this kinase are the microtubule associated protein, MAP2, synapsin 1, synGAP, the AMPA receptor and the NMDA receptor (Fukunaga et al., 1993, Tan et al., 1994, Omkumar et al., 1996, Nayak et al., 1996, Barria et al., 1997, Chen et al., 1998). MAP2 is a protein that interacts with cytoskeleton proteins such as actin filaments and microtubule in a CaMKII phosphorylation dependent manner. Activation of NMDA receptors causes alterations in the phosphorylation state of MAP2 (Halpain and Greengard, 1990, Quinlan and Halpain, 1996) which may play a role in remodeling of the cytoskeleton. The phosphorylation of presynaptic protein synapsin 1 by CaMKII may also be relevant in plastic changes (Burgoyne and Morgan, 1995).

Protein kinase A (PKA) is a serine / threonine kinase activated by cyclic AMP, the level of which is controlled by the continuous action of adenyl cyclases and cAMP phosphodiesterases. Adenyl cyclases can be sensitive to calcium / calmodulin (Xia and Storm, 1997). Cyclic adenosine mono phosphate (cAMP) is elevated in an NMDA dependent manner after tetanic stimulation and indicates the involvement of PKA in LTP (Chetkovich et al., 1991). Genetic ablation of the neurospecific adenyl cyclase type 1 in mice show deficits in LTP with potentiation developing slowly and reaching a reduced level compared to wild type mice (Wu et al., 1995). Activation of PKA increases non-NMDA mediated currents (Greengard et al., 1991, Wang et al., 1991) and the phosphorylation of GluR1 AMPA receptor subunit (Roche et al., 1996) implying that this molecule is involved in the process of LTP.

Evidence for the involvement of the mitogen–activated protein kinase (MAPK) cascade in LTP has also been presented. This kinase cascade is involved in the

processes of cell proliferation and differentiation, but the continued expression and activity of this pathway in post-mitotic neurones of the adult CNS indicates a further function. MAPKs are a family of serine / threonine protein kinases that act as primary effectors of growth factor signalling cascades. One isoform, p42 MAPK, is activated in response to NMDA receptor activation and high frequency stimulation in area CA1 of the hippocampus (English and Sweatt, 1996). Inhibitors of this kinase cascade block the induction of LTP and have no effect on established LTP (English and Sweatt, 1997).

This pathway can also be activated by ras ($p21^{ras}$) activation (see Figure 2). Ras proteins have been shown to play a crucial role in receptor activated signal transduction pathways (such as for the neural cell adhesion molecule, NCAM, Schmid et al., 1999). Growth factor receptors and non-receptor tyrosine kinases can both activate $p21^{ras}$ through the Grb2-SOS complex. The active $p21^{ras\text{-}GTP}$ complex initiates a phosphorylation cascade of: Raf / MAPKK / MAPK which modulates the levels of translation and transcription in the cell providing a pathway to initiate protein synthesis after LTP induction.

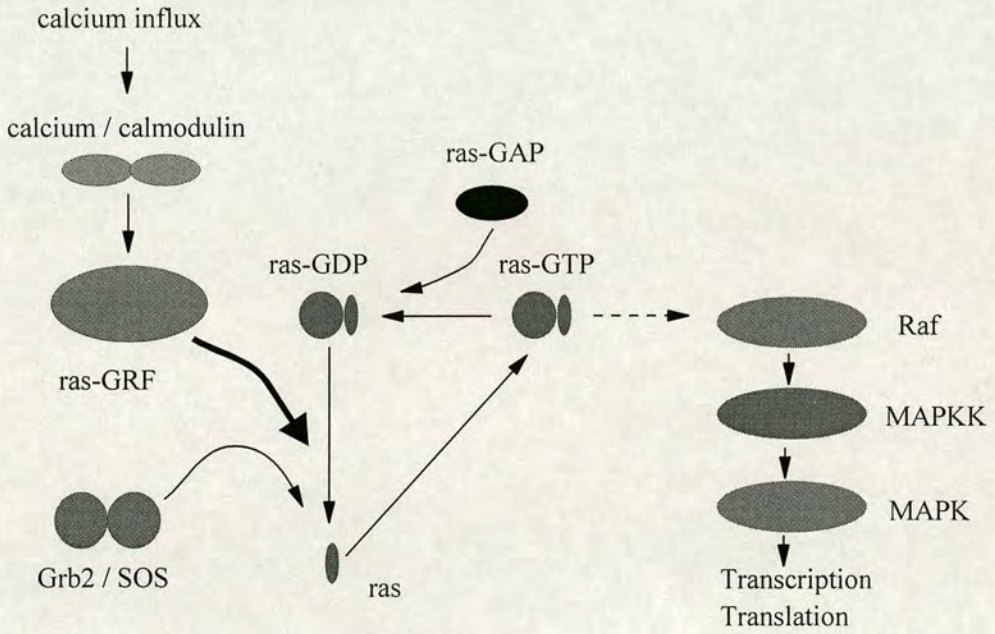


Figure 2 – The ras activation of the MAPK pathway
(See text and chapter four for details)

There is evidence that protein synthesis is necessary for prolonged LTP. Studies using anisomycin (an inhibitor of mRNA translation) reduced the duration of LTP (which can be maintained for several days *in vivo*), to 3-6 hours (Frey et al., 1989). The effect of actinomycin D, an inhibitor of gene transcription, has also been studied, and has no effect on LTP for the first three hours (Frey et al., 1996). Evidence for a role of protein kinases in late stages of LTP also exists, as there is an up-regulation of CaMKII mRNA and a down-regulation of the β isoform of PKC mRNA. The synthesis of proteins required for a long lasting synaptic change may occur locally at the dendritic site or presynaptically in the nerve terminal, however, no evidence has been reported to support either of these ideas. The principle site for protein synthesis is the cell soma, but this raises the problem of input-specificity (seen in all forms of Hebbian long-term potentiation). How do proteins synthesised in the cell soma target the specific synapse at which long-term potentiation occurs? Frey and Morris proposed a hypothesis for this process, termed synaptic tagging (Frey and Morris, 1997, 1998). The authors proposed that a short lived (2-3 hours) protein synthesis independent 'synaptic tag' is established following LTP induction, which sequesters the relevant proteins for the development of longer lasting changes. Through elegant dual pathway late phase LTP experiments, the authors demonstrate that this 'synaptic tag' shows input-specificity to a particular synapse, is short lived and is not generated in conditions where short-term potentiation is induced. In this model, the 'synaptic tag' has a postsynaptic locus, whilst a model involving the development of a presynaptic 'synaptic tag' has also been proposed (Routtenberg, 1999).

The non receptor tyrosine kinases of the src family are also fundamentally involved in LTP and are examined in detail below.

1.7. Tyrosine kinases

Tyrosine kinases fall into two structurally distinct categories:

1. Receptor tyrosine kinases that transduce signals from growth and neurotrophic factors, e.g. nerve growth factor receptor (NGFR) and platelet derived growth factor receptor (PDGFR)
2. Non-receptor tyrosine kinases associated with the cytoplasmic side of the plasma membrane (e.g. fyn, src, and yes).

Many lines of evidence have implicated both forms of tyrosine kinases in the induction and maintenance of hippocampal LTP (O'Dell et al., 1991, Grant et al., 1992, Kojima et al., 1997, Lu et al., 1999, Tezuka et al., 1999, Kang and Schuman, 1995, Korte et al., 1996, Patterson et al., 1996, Abe et al., 1991). For the purpose of this thesis, attention is focused on the src family of non-receptor tyrosine kinases.

1.7.1. The src family of non-receptor tyrosine kinases

The gene for the non-receptor tyrosine kinase src was first identified as a proto-oncogene of the Rous sarcoma virus (v-src, Martin, 1970, Vogt, 1971, Toyoshima and Vogt, 1969). A homologous non-transforming cellular form of src, designated $pp60^{c\text{-}src}$ (c-src), was later discovered (Spector et al., 1978, Stehelin et al., 1976). This was the first member of the src family of non-receptor tyrosine kinases, which now includes many members (e.g. $p59^{fyn}$, $p59^{hck}$, $p62^{yes}$, $p56^{lck}$, $p56^{lyn}$, $p55^{fgr}$ and $p55^{blk}$). Fyn, originally designated as syn (*s*rc / *y*es related *n*ovel gene), was identified as a novel member of the src tyrosine kinase family by molecular cloning in the human genome (Semba et al., 1986). All members share similar structural components; an N terminal domain (including a unique region of 70-82 residues), a protein-protein binding domain specific for proline-rich motifs (SH3 domain, src-homology), phosphotyrosine residues located next to hydrophobic residues (SH2

domain), followed by the highly conserved kinase domain (SH1) responsible for the enzymes catalytic activity (see Figure 3).

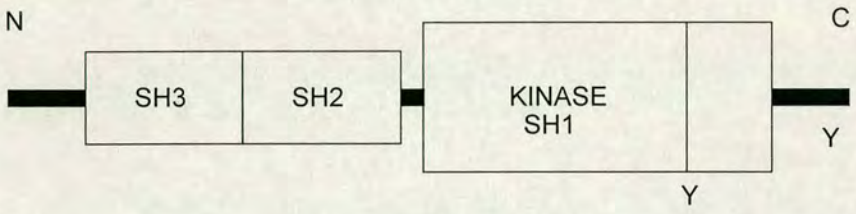


Figure 3 - Diagrammatic structure of the src family of kinases
(see text for details)

The N terminal is modified by the addition of a myristic acid moiety allowing an interaction with the plasma membrane. The unique region follows the small N terminal region and contains sites that associate with surface receptors (such as CD4 and CD8 in src), sites of serine phosphorylation by protein kinase C (Ser-12 in src) and cAMP-dependent kinases (Ser-17 in src). Fyn can also be phosphorylated on serine residues, although the sites are unknown.

The SH2 and SH3 domains are well conserved and found in several other cellular proteins (including phospholipase C γ, rasGAP and tyrosine phosphatases). SH2 and SH3 domains appear to mediate protein-protein interactions with other intracellular proteins. The SH2 domain of the src family kinases binds to phosphotyrosine containing sequences with different optimal binding sequences for each family member. The optimum binding sequence for SH2 domains of src family kinases is pTyr-Glu-Glu-Ile (YEEI) and the specificity of binding for each member depends on the residues adjacent to the phosphotyrosine (pTyr, Songyang et al., 1993). The SH3 domain is found in a variety of other proteins (such as myosin IB, PLCγ and α-spectrin) and is often together with SH2 domains.

SH3 domain interactions have been implicated in src kinase binding to PI 3-kinase (Prasad et al., 1993) and the regulation of Ras guanine nucleotide exchange (Simon et al., 1993, Olivier et al., 1993). This interaction is mediated by SH2 and SH3 binding the Ras guanine nucleotide releasing protein, Son of sevenless (SOS), providing a means to couple protein tyrosine kinases to $p21^{ras}$ activation. SH3 interactions have also been implicated with the Rho / Rac GTP-binding proteins, responsible for events such as membrane ruffling and the formation of focal contacts (Ridley and Hall, 1992).

Following the SH2 domain is the highly conserved kinase domain of about 300 amino acids that exhibits some 80% homology between family members. An autophosphorylation tyrosine residue is present in the kinase domain, which is important for the regulation of binding and activation of these kinases (Tyr-416 for src, Tyr-420 for fyn). The C-terminal domain also includes a negative regulatory site (Tyr-527 for src, Tyr-531 for fyn). Phosphorylation of this residue inhibits catalytic activity and is mediated by the cytosolic tyrosine kinase Csk (C-terminal src kinase, Nada et al., 1991, Partanen et al., 1990) whilst dephosphorylation stimulates kinase activity and may be mediated by protein tyrosine phosphatases (PTPases) such as CD45 (Ostergaard et al., 1989, Mustelin et al., 1989). The transforming v-src lacks this C-terminal tyrosine and mutation of this residue induces constitutive kinase activity (Cartwright et al., 1987, Piwinica-Worms et al., 1987). A current model postulates that these kinases are regulated by binding of the phosphorylated C-terminal tyrosine to the SH2 region of the kinase, inhibiting activity (Matsuda et al., 1990). In this inhibited configuration the kinase rests inactive until dephosphorylation of the C-terminal tyrosine allows unfolding and subsequent binding of substrate molecules.

Alternative splicing of the src, fyn and lyn genes generates different isoforms of these kinases. Two isoforms of fyn (T and B) are formed from mutually exclusive splicing of the seventh exon. $p59^{fynT}$ (exon 7B) is expressed exclusively in T lymphocytes and $p59^{fynB}$ (exon 7A) expressed primarily in brain (Cooke and Perlmutter, 1989). Murine c-src gene can encode three proteins, two of which are

expressed in neural cells and a third expressed ubiquitously, differing only in their SH3 regions (Levy et al., 1987, Tanaka et al., 1987). Two isoforms of the lyn gene ($p53^{lyn}$ and $p56^{lyn}$) differ in their N terminal regions and are concordantly expressed in cells.

Fyn is expressed throughout the brain during development and in the adult its highest levels of expression are seen in the hippocampus (Umemori et al., 1992, Yagi et al., 1993, Bare et al., 1993). Src is highly expressed at embryonic stages where it is concentrated in neuronal growth cones and process rich regions (Sorge et al., 1984, Maness et al., 1988) and expression persist in adults, the highest levels seen in the hippocampus, pyriform cortex, and the neocortex (Hirano et al., 1988, Brugge et al., 1985, Cotton and Brugge, 1983). These expression patterns are in contrast to other src family members such as yes and lyn, which are expressed only in adult stages (Umemori et al., 1992, Sudol, 1988). It is interesting to note that src, fyn and yes regional expression patterns in brain overlap almost entirely, suggesting that these kinases can compensate for one another (Zhao et al., 1991). These expression patterns provide evidence for a role of the src family of tyrosine kinases in neural tissue, distinct from their reported involvement in oncogenic and lymphatic systems.

Genetic knockout studies of the src family tyrosine kinases have also implicated these kinases in synaptic plasticity. Mice lacking fyn kinase ($fyn^{-/-}$, fyn knockouts) display impaired LTP induction, disrupted hippocampal morphology and impaired performance in the Morris water maze test of spatial learning (Grant et al., 1992). These effects were specific to fyn knockouts, and not seen in src, yes and abl knockout mice. LTP was not abolished in these animals, indicating that fyn in not an absolute requirement for the induction of LTP. Tetanic stimulation (2x100Hz) at 25% of the maximal fEPSP size gave little or no potentiation (108% of control response) in $fyn^{-/-}$ mice, whilst inducing robust LTP in wild type mice (149% of control response). Stimulation at 75% of the maximal fEPSP size produced robust LTP in $fyn^{-/-}$ mice (133% of control response), though this was at a reduced level to that seen in wild type mice (168% of control response). These results indicate that fyn modulates the threshold of LTP induction.

There are two distinct forms of fyn knockout mice. One which was created by replacing the first coding exon of the fyn gene with a neo expression cassette ($fyn^{-/-}$, Stein et al., 1992). The other fyn knockout mice was independently derived by inserting the β-galactosidase gene (*lacZ*) into the fyn gene locus (fyn^{Z} / fyn^{Z}, Yagi et al., 1993).

The Grant et al. (1992) study and the animals used in this thesis are derived from the Stein mice ($fyn^{-/-}$). Several phenotypes have been described in the $fyn^{-/-}$ mice and are detailed briefly. Mice deficient in the thymic isoform of fyn ($p59^{fynT}$) show no overt phenotype, but display defective T cell receptor signalling. Mice lacking both isoforms of fyn also show defective thymocyte signalling but displayed normal splenic T cell signalling (Stein et al., 1992). The lack of any overt phenotype in both of these experiments shows that fyn is not essential during embryonic development. This however is attributed to the compensation of the loss of fyn by one of the other src family members. Src, fyn and yes all show similar tissue distribution, all are expressed in a wide variety of cell types and are especially enriched in the brain and platelets (Bolen, 1991). The fact that these kinases are all expressed in the same cell types indicates that compensatory effects may occur. This is further supported by biochemical analysis showing the association of multiple kinases with common signal transduction pathways, suggesting that these kinases are interchangeable (Kypta et al., 1990, Burkhardt et al., 1991, Huang et al., 1991, Sugie et al., 1991). An overt phenotype is seen in mice lacking Csk, the tyrosine kinase responsible for the negative regulation of src family members (Nada et al., 1991), which leads to neural tube defects and embyronic lethality at E9-10 (Imamoto and Soriano, 1993). This shows that uncontrolled activation of src family kinases can cause severe developmental problems, even though the individual members are not essential for normal embryogenesis. Fyn also appears to be involved in the process of epileptogensis, indicated by retardation in the rate of kindling (Cain et al., 1995).

A significant amount of data has been produced concerning mice with a *LacZ* gene inserted into the fyn locus (Yagi et al., 1993). Fyn^{Z} / fyn^{Z} mice show abnormal phenotypes, several which may be linked to a neural function for fyn tyrosine kinase.

Animals display impaired suckling behavior and abnormal organisation of the modified glomerular complex (MGC) in the olfactory bulb (Yagi et al., 1993). Evidence for abnormal synaptic transmission has been detailed in slice preparations from the olfactory bulb, in which fyn^Z / fyn^Z mice displayed a decreased sensitivity to GABA antagonists bicuculline and picrotoxin. A form of NMDA receptor dependent LTP was also found to be impaired in this brain region in these animals (Kitazawa et al., 1998). Fyn is also important for the initial events in axon myelination. A 50-60% reduction in the levels of myelin was found in fyn^Z / fyn^Z mice, in absence of a reduction in the amount of neuronal material (Umemori et al., 1994). A recent report indicates that fyn stimulates the promoter activity of the myelin basic protein gene around sevenfold, indicating a role for fyn signalling in gene induction (Umemori et al., 1999).

Ethanol administration enhances tyrosine phosphorylation of the NMDA receptor subunit NR2B (but not NR2A) in + / fyn^Z mice, but not in fyn^Z / fyn^Z mice. NMDA receptor mediated field EPSPs (fEPSP) in hippocampal slices were initially depressed upon application of ethanol, however the development of acute tolerance (recovery of the amplitude of NMDA receptor fEPSPs) only occurred in + / fyn^Z mice. This implies that tyrosine phosphorylation of NR2B is involved in the development of acute tolerance to ethanol administration and involves the action of fyn (Miyakawa et al., 1997, Yagi, 1999). Another recent study detailed the involvement of fyn in the phosphorylation of the NR2A subunit in association with PSD-95 binding. NR2A and NR2B subunits from fyn knockout (fyn^Z / fyn^Z) mice were shown to have a reduced level of tyrosine phosphorylation indicating fyn is important but not the only kinase involved. When fyn and NR2A subunits were co-expressed in 293T cells, tyrosine phosphorylation was induced and expression of the NMDA receptor binding protein PSD-95 further increased this phosphorylation. Fyn was shown to bind the third PDZ domain of PSD-95 *in vitro* and *in vivo* (along with other src family members src, yes and lyn) through its SH2 domain (Tezuka et al., 1999).

Further evidence of the involvement of fyn and src tyrosine kinases in synaptic functions has come from electrophysiological and biochemical studies. The induction of NMDA receptor dependent LTP in area CA1 of the hippocampus can be blocked by tyrosine kinase inhibitors (O'Dell et al., 1991) demonstrating that this family of kinases play an important role in synaptic transmission in the CNS. NMDA receptor activation stimulates a tyrosine kinase that phosphorylates a serine / threonine kinase (MAP-2, Bading and Greenberg, 1991) indicating a role for tyrosine phosphorylation in an NMDA receptor dependent signalling cascade. Depolarisation, glutamate application, mGluR agonists, AMPA receptor agonists, muscarinic receptor activation and α1-adrenergic receptor activation (bringing about an increase in intracellular calcium concentration) in both hippocampal slices and primary culture also stimulate tyrosine phosphorylation of cellular proteins (Siciliano et al., 1994). Direct evidence of NMDA receptor modulation by tyrosine kinases has been reported using tyrosine kinase and phosphatase inhibitors. Intracellular injections (using the perforated patch technique) of the tyrosine kinase inhibitors lavendustin A and genistein produced a significant decrease in NMDA receptor mediated currents and intracellular calcium flux. Conversely, intracellular administration of $p60^{src}$ potentiated NMDA receptor mediated currents approximately twofold. A potentiation of NMDA receptor currents was also seen upon the intracellular administration of sodium orthovanadate, a protein tyrosine phosphatase inhibitor, indicating that the NMDA mediated current can be modulated in a bi-directional manner by phosphorylation and dephosphorylation (Wang and Salter, 1994).

Biochemical studies have indicated that the NMDA receptor subunits NR2A and NR2B are substrates for fyn tyrosine kinase (Suzuki and Okumura-Noji, 1995) and tyrosine phosphorylation of these subunits has been reported *in vivo* (Lau and Huganir, 1995). The major tyrosine phosphoprotein found in postsynaptic densities is the NR2B subunit (Moon et al., 1994). Recombinant NMDA receptors transiently expressed in human embryonic kidney cells showed a different regulation by src and fyn kinases. In this study only cells expressing NR1-NR2A subunits showed enhanced currents in the presence of src and fyn, although mutational analysis in this

report indicates that the site of action of these kinases lies in the C-terminal tail of the NR2 subunits (Kohr and Seeberg, 1996). In a further study, Chen and Leonard stimulated endogenous insulin receptors to activate tyrosine kinases in NMDA subunit expressing oocytes. Enhanced NMDA mediated responses were seen in NR1-NR2A, NR1-NR2D heteromeric and NR1 homomeric channels (Chen and Leonard, 1996). This result is suprising as no tyrosine phosphorylation sequences have been identified in the NR1 subunit. Two independent studies reported that tyrosine phosphorylation of the NR2B subunit was enhanced in dentate gyrus LTP. This effect was apparent after several minutes, persisted for several hours after induction and subsided after 24 hours. This may indicate a role for tyrosine phosphorylation in the maintenance stage of LTP rather than the induction stage (Rosenblum et al., 1996, Rostas et al., 1996).

Single channel patch clamp analysis of NMDA channels from cultured rat neurones and mEPSC analysis in hippocampal slices demonstrated regulation by src, which was dependent on a specific region of the unique domain of src. Through the use of a src specific antibody, this tyrosine kinase was co-precipitated with the NMDA receptor indicating that there may be a physical link between the two (Yu et al., 1997). The same group provided further evidence of the role of tyrosine kinases in LTP. Again using src specific peptides and antibodies, they demonstrated that the rise in the NMDA receptor mediated component was calcium independent (thus mediated directly through src). The AMPA receptor mediated component (in conditions where intracellular calcium buffering was low) was also enhanced by src activating peptides but in a calcium and NMDA receptor dependent manner. This implies that there is a 'fast' src mediated enhancement of NMDA receptor current that enhances the calcium influx and triggers the downstream signalling cascade (Lu et al., 1998).

The GluR1 AMPA receptor subunit can be tyrosine phosphorylated *in vitro* (Moss et al., 1993) and receptor tyrosine kinases (BDNF receptor) have been shown to enhance AMPA mediated synaptic transmission (Kang and Schuman, 1995). Recent evidence has demonstrated that BDNF and PDGF receptor activation enhances the

expression of AMPA receptor subunits GluR1, GluR2 and GluR3 by a src family tyrosine kinase dependent pathway. Interestingly, fyn knockout mice show a reduced level of AMPA receptor subunit expression (Narisawa-Saito et al., 1999).

The recent discovery of the formation of a macromolecular receptor complex, linking ion channel subunits to scaffolding proteins (such as the PSD-95 / NMDA receptor and GRIP / AMPA receptor complexes) indicates that signalling molecules and regulatory molecules can be localised at the site of ion channel subunits (see above). Fyn has now been shown to phosphorylate the NMDA receptor subunit NR2A but only when in association with PSD-95. This effect is not seen on NR2B subunits, perhaps indicating that the localisation of a regulatory molecule may impart its substrate specificity (Tezuka et al., 1999). This study also demonstrates a reduced level of NR2A tyrosine phosphorylation in fyn knockouts. The large body of evidence demonstrating a role for the src family of tyrosine kinases in glutaminergic synaptic transmission and plasticity has maintained interest in this group of kinases.

It should also be noted that the src family of tyrosine kinases have been implicated in the phosphorylation mechanisms of other receptor types. $GABA_A$ type inhibitory receptor subunits $\beta 1$ and $\gamma 2L$ are tyrosine phosphorylated when co-expressed with src in mammalian cells. This phosphorylation enhanced whole cell GABA mediated currents, in both mammalian cells and primary neuronal cultures (Moss et al., 1995). The modulation of GABA mediated currents by src family kinases has also been reported in primary neuronal cultures (expressing native $GABA_A$ receptors, Wan et al., 1997, Herron and Grant, 1997). This indicates that the src family of tyrosine kinases can modulate synaptic inhibition as well as excitation.

The nicotinic acetylcholine receptor (nAChR) is also closely associated with src family kinases. Tyrosine phosphorylation of the nAChR is present both *in vitro* and *in vivo* and regulates the rate of receptor desensitisation and the clustering of the receptors (Qu et al., 1990, Wallace et al., 1991). Both fyn and fyk (a novel member identified in *Torpedo*) binds to the nAChR through their SH2 domains (Swope and Huganir, 1993, 1994) and mediate this phosphorylation. The clustering property of

fyn, fyk and also src is mediated by a cytoskeletal protein rapsyn, which activates the src family kinases inducing phosphorylation of the nAChR and its binding to the cytoskeleton (Mohamed and Swope, 1999).

1.7.2. Focal adhesion kinase

Biochemical analysis of $fyn^{-/-}$ mice showed nine proteins hypo-phosphorylated on tyrosine residues (Grant et al., 1995). One of these proteins was identified as focal adhesion kinase (FAK), a non-receptor tyrosine kinase whose structure is unique among the families of protein tyrosine kinases as it contains no SH2 or SH3 domains (Schaller & Parsons, 1993). In non-neural cells, the expression of this kinase is restricted to focal contacts (an integrin linked contact between the cell membrane and the extracellular matrix, Hildebrand et al., 1993, Yamada and Geiger, 1997, Richardson and Parsons, 1995, Frish et al., 1996). However, in neural tissue FAK is expressed throughout axons, dendrites and the intermediate filament cytoskeleton of astrocytes (Grant et al., 1995). FAK is expressed at high levels during neural development and in the adult brain expression is particularly high in neurones of the hippocampus and cerebral cortex (Burgaya et al., 1995, Grant et al., 1995). These data indicate that FAK may have a specific neuronal function, aside from its role in maintaining focal adhesions as found in other cell types. FAK activity is regulated by both fyn and src (Calalb et al., 1995, Kanazawa et al., 1996), and appears to be a good candidate as a component of a fyn dependent pathway. Src and fyn have been shown to form stable complexes with FAK (Cobb et al., 1994). As synaptic remodelling is a possible mechanism by which changes in synaptic efficacy could occur following LTP, the fyn-FAK pathway might provide a suitable signalling mechanism, however the subcellular distribution of this kinase hints at a novel role in neurones. To determine the role of FAK in neural tissue, $FAK^{-/-}$ knockout mice were produced (Llic et al., 1995a), however the homozygous mutation is lethal at E8.5. FAK-deficient cells in culture show reduced cell motility and enhanced focal adhesion formation (Llic et al., 1995b). FAK heterozygous mice ($FAK^{+/-}$) are viable and show no major abnormalities. In these mice, the level of FAK activity should be

reduced by a considerable amount. To further reduce the functional levels of FAK activity, these mice were crossed with fyn homozygous mice ($fyn^{-/-}$). Fyn regulates the kinase activity of FAK; thus the cross should reduce activity further as this protein is hypo-phosphorylated in $fyn^{-/-}$ mice. These mice were viable and displayed no gross developmental or behavioral abnormalities. Details are presented of LTP experiments in these mice (Chapter 3). Two alternatively spliced forms of FAK exist, one of which is neuronal specific, termed FAK (+), which displays an increased autophosphorylation activity (Burgaya et al., 1997, Derkinderen et al., 1996). A second member of the FAK family of tyrosine kinases, PYK2 has been identified. PYK2 can tyrosine phosphorylate the Kv1.2 potassium channel and activate the MAPK kinase cascade in a calcium dependent manner (Lev et al., 1995). The existence of a brain specific isoform, regulation by src family kinases, regulation by calcium and the finding that FAK is distributed throughout axonal and dendritic regions in neurones suggests a role for this isoform in synaptic function.

The cellular and molecular mechanisms of induction and maintenance of NMDA receptor dependent LTP in area CA1 of the hippocampus are at present poorly understood. The recent advancement of molecular techniques have led to a large body of evidence implicating hundreds of molecules, as well as the components of ion channel receptors themselves, in the regulation of LTP (Sanes and Lichtman, 1999). However, no consensus on their underlying molecular mechanisms has emerged. The molecules that are particularly relevant in this area are those which modulate ion channel receptors directly and molecules involved in the signalling pathways downstream of these receptors. The function of the src family of tyrosine kinases and their interaction with intracellular signalling pathways are investigated in this thesis, using electrophysiological recordings made in genetic knockout animals.

Chapter II

General Methods

2.1. Experimental animals

Mice from either a hybrid strain of C57BL/6 x 129/Sv or the 129/Sv inbred strain were used in experiments investigating short and long-term plasticity in area CA1 of the murine hippocampus. Animals were over the age of 14 weeks for all experiments (except where stated). Stocks of mice were maintained by backcrossing heterozygous or homozygous brother / sister pairs. Wild type littermates (identified by polymerase chain reaction (PCR) analysis of tail samples) generated from the heterozygous matings were used as a control group. The pure strain of 129/Sv animals were sourced from The Jackson Laboratory, Maine, U.S.A. (129/Sv-Fyn<tm1Sor>, Catalogue Number JR2271). Four $fyn^{-/-}$ mice (3 males, 1 female) were obtained and backcrossed to generate a new colony of 129/Sv animals. Mice generated from the 129/Sv inbred strain will be denoted as $fyn^{129/Sv-/-}$ or wild type 129/Sv mice to distinguish them from the hybrid C57BL6 x129/Sv ($fyn^{-/-}$) strain.

The mice were bred, housed and attended to by the staff in the Centre for Genome Research Animal House. Ras-GRF mutant mice were generated at The European Molecular Biology Laboratory, Heidelberg, Germany and subsequently shipped to The Centre for Genome Research. No subsequent breeding was performed. The study of Ras-GRF knockout mice was performed in collaboration with Ricarrdo Brambilla from the Europe Molecular Biology Laboratory (EMBL), Heidelberg. Hippocampal LTP experiments were performed by the author of this thesis. Dr Paul Chapman (University of Wales, Cardiff) was responsible for the amygdaloid electrophysiology and all other reported details are taken from the subsequent publication (Brambilla et al., 1997).

$Fyn^{-/-}$ and $fyn^{-/-}$ / $FAK^{+/-}$ animals (and littermate controls) were maintained on a hybrid C57BL/6 x 129/Sv genetic background. $Fyn^{129/Sv-/-}$ animals (and littermate controls) were maintained in an inbred 129/Sv genetic background. Ras-GRF mutant mice were either from a C57BL/6 or 129/Sv inbred genetic background. Certain experiments were performed in a blind fashion, specifically those involving the Ras-GRF mice, the $fyn^{-/-}$ / $FAK^{+/-}$ LTP study, the replication of the Grant et al LTP

studies (in all conditions) and the preliminary PPF studies. All other experiments were performed with the knowledge of the animals genotype.

2.2. Artificial cerebrospinal fluid (ACSF) composition

During the course of these experiments several compositions of ACSF were used to perfuse hippocampal slices. These are detailed in Table 1. All ACSF solutions were constantly gassed with 95% O_2 / 5% CO_2. $CaCl_2$ was added separately (after the solution had time to equilibrate with the oxygen / carbon dioxide mixture) from a 1M stock solution to avoid re-precipitation. The 'standard' (see Table 1) solution was used for slice preparation except in one case where a modified cutting solution ('Sucrose' see Table 1) was used in which sodium chloride was replaced by D-sucrose. Extracellular and intracellular recordings were made in the 'standard' ACSF and the 'Grant et al. 1992' ACSF that has higher potassium, higher sodium, lower calcium and higher magnesium ion concentrations than the 'standard' solution. The 'Grant et al. 1992' solution was only used in one series of experiments (see 3.3.1), all other experiments were performed using the 'standard' ACSF.

2.3. Preparation of transverse hippocampal slices

Mice were anaesthetised with halothane to the point of abolition of the hind-paw withdrawal reflex. Spinal dislocation and decapitation with large scissors followed. A rapid craniotomy was performed, the scalp and muscles of the neck were removed and the remains of the spinal column were trimmed with fine scissors. The parietal and frontal bones were cut sagitally along the midline and prised to the side using blunt ended tweezers. During this procedure care was taken to ensure minimum damage to the cortex and underlying structures. The dura was cut and peeled to the edges of the skull after which a small spatula was used to lift the brain and sever the cranial nerves. The brain was removed and transferred to a chilled (1- 4°C) beaker of gassed ACSF for 30 seconds. A scalpel cut down the midline was used to separate

Table 1 – ACSF composition

Solution	*NaCl (mM)*	*KCl (mM)*	*$MgSO_4$ (mM)*	*NaH_2PO_4 (mM)*	*$NaHCO_3$ (mM)*	*$CaCl_2$ (mM)*	*Glucose (mM)*	*Sucrose (mM)*
Standard	119.0	2.5	1.3	1.0	26.2	2.5	11.0	-
Grant 1992	124.0	4.4	2.0	1.0	25.0	2.0	10.0	-
Low Calcium	119.0	2.5	2.5	1.0	26.2	1.3	11.0	-
High Calcium	119.0	2.5	0.5	1.0	26.2	4.5	11.0	-
Sucrose	-	2.5	10.0	1.2	26.0	2.0	10.0	252.0

the two cerebral hemispheres. The hippocampus from each hemisphere was dissected out with a blunt ended spatula. Both hippocampi were placed on ASCF moistened filter paper (Whatman No 3) on the stage of a gravity tissue chopper (Stoelting Company, U.S.A.) in the appropriate orientation for transverse slicing. Slices were cut to a thickness of 400μm by moving the stage relative to the blade with an in-built micrometer. These slices were transferred, using a fine paintbrush, to a petri dish containing gassed ACSF, and left to equilibrate for 1-2 hours before recordings commenced. In some cases slices were directly transferred to an interface recording chamber to recover.

2.4. Recording chamber

The majority of experiments were performed in a submerged type of recording chamber. This design is described in detail below and shown schematically in Figure 4.

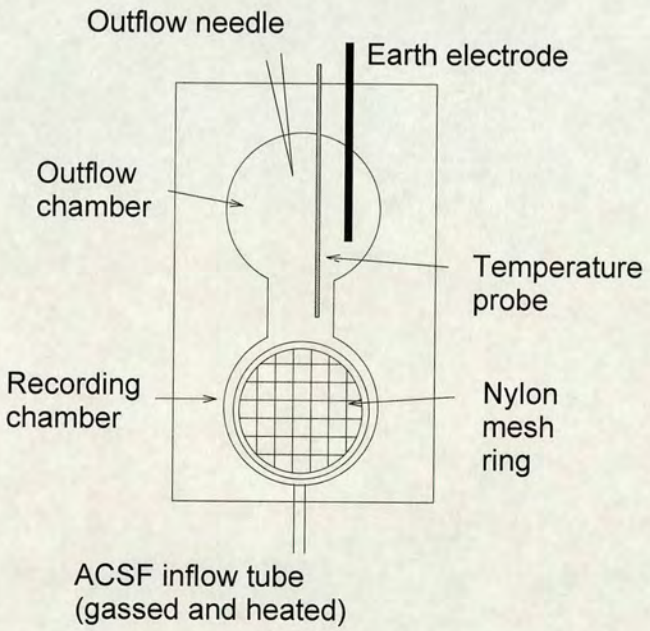


Figure 4 – Schematic diagram of the submerged recording chamber

(See text for details)

Hippocampal slices were placed in the central recording chamber and held down by means of a small plastic ring (approximately 400 - 500μm thick) covered with nylon mesh. The flow rate of oxygenated ACSF was controlled by means of a peristaltic pump (Harvard Apparatus, U.S.A.) feeding the initial chamber at a rate of 3 - 4 $ml.min^{-1}$. ACSF was removed from the outflow chamber through a syringe needle connected to a vacuum pump (Charles Austin Pumps Ltd., U.K.). All experiments were performed at 29 ± 1°C, which was maintained by means of a heating tape and water jacket surrounding the inflow tube. A digital thermocouple (Digitron, RS Components, U.K.) recorded the temperature directly from the recording chamber allowing accurate control of ACSF temperature surrounding the slices. Two other recording chambers were used, both modified versions of the interface recording chamber designed by Spencer et al. (1976). Slice preparation and maintenance is reviewed in Aitken et al. (1995) and Lipton et al. (1995).

2.5. Microelectrodes

Microelectrodes were prepared from standard or thin walled borosilicate glass (Clark Electromedical Instruments Ltd., UK) using Flaming Brown P-87 Microelectrode puller (Sutter Instruments, UK). Extracellular microelectrodes were filled with 1M sodium chloride and had resistances ranging from 2 – 5 MΩ. Intracellular microelectrodes were back filled with 2M potassium methyl-sulphate and had resistances ranging from 40 – 80 MΩ. All electrode filling solutions were made with ultra-high purity distilled water and filtered through 0.22μm sterile syringe filters. Filled microelectrodes were mounted in Perspex electrode holders containing a silver / silver chloride coated wire (Axon Instruments, USA). The electrode holder was attached to the unity gain headstage of a high impedance Axoclamp-2B amplifier (Axon Instruments, USA). The headstage was attached to a piezo electric step control (Significat, Digitimer, U.S.A.) allowing the advancement of the recording electrode into the hippocampal slice in 2μm steps. A silver / silver chloride coated bath reference electrode was connected to the head stage and grounded though the Axoclamp-2B.

2.5.1. Electrode positioning

Extra-cellular recording microelectrodes were positioned near the middle of *stratum radiatum* in the CA1 pyramidal subfield by visualisation with a binocular dissection microscope (Nikon SMZ-2B, Japan). Intra-cellular recording electrodes were manually placed in the centre of *stratum pyramidale* (CA1 pyramidal cell body layer) and moved vertically by a piezo electric step controller (Significat, Digitimer, U.S.A.). The Schaffer collateral-commissural fibre input to CA1 pyramidal neurones was stimulated by placing bi-polar steel stimulating electrodes in *stratum radiatum* at the border of the CA3/CA2 subfields. The distance between the recording and stimulating electrodes was variable, as was the depth of the recording electrode. This allowed the optimum field EPSP to be recorded in each slice. At all times however, the tip of the recording electrode was not placed in the upper or lower 50μm of the slice, which is prone to damage during the slicing procedure.

2.6. Intracellular recordings

Microelectrodes for impalement of CA1 neurones were lowered manually into the surface of the slice. Extracellular potentials recorded in the circuit between the microelectrode and bath reference electrode were compensated for using an offset potentiometer. Square DC pulses of 200 pA in amplitude (100ms, 2Hz) were injected through the microelectrode and the bridge was balanced via a potentiometer, providing an estimation of the electrode resistance. The electrode was advanced using the motorised step controller until a negative deflection of the voltage trace (seen on the oscilloscope) indicated the electrode was against the neuronal membrane. A brief injection of high amperage oscillatory current was used to 'buzz' the electrode tip into the cell, sometimes combined with an additional 2μm step. The mechanism by which impalement occurs is not known (Silinsky, 1992), but is thought to compromise the integrity of the membrane allowing the tip of the electrode to enter the cell. After impalement, a short period of constant hyperpolarising current (-100 to -500 pA) was injected into the cell to allow the

membrane to stabilise. The oscilloscope trace shifted by the number of millivolts (mV) corresponding to the cell's resting membrane potential which was displayed on the Axoclamp 2B digital voltmeter.

2.7. Recording apparatus

Signals from the headstage were sent to the Axoclamp 2B amplifier through the microelectrode 1 (ME1) port for use in 'bridge mode' (both extracellular and intracellular). Current and voltage (x10) outputs (filtered at either 1kHz or 3kHz) were sent to a digital storage oscilloscope (TDS-310, Tektronic Inc.) where they were monitored continuously. The voltage and current outputs were sent to either a PP50-LAB Patch Panel (Warner Instruments Corp., U.S.A.) analogue-to-digital (A/D) interface or a Digidata 1200 A/D interface (Axon instruments, USA) connected to a Pentium 90 (Gateway, USA) personal computer. Enroute to the interface, secondary amplification was provided by a variable gain DC amplifier (Model 210A, Brownlee Precision, U.S.A.) for optimal use of the A/D range. Current commands were generated from the computer using pClamp software (Axon Instruments, USA). Triggering pulses and stimulation pulses were controlled using a Master 8 pulse generator (A.M.P.I., Israel), connected to the computer and an isolated constant voltage / constant current stimulator (Iso-Flex, A.M.P.I., Israel). Acquisition, storage and analysis of data were performed by a software program 'Mintra' (David Selig, 1993-94) or 'LTP110x' (William Anderson, 1991-98) and by commercially available software (pClamp series, Axon Instruments). All data were stored and analysed in real time on the hard drive of an IBM compatible PC and subsequently backed up to magnetic tape or CD-ROM.

2.8. Long term potentiation protocol

Synaptic potentials were evoked by stimulating the Schaffer collateral pathway at a frequency of 0.2Hz and gradually increasing the stimulus voltage applied until a

suitable fEPSP was generated. The initial slope of fEPSP was measured as an indication of the AMPAR mediated component of the fEPSP and as a measure of synaptic efficacy. The synaptic potentials evoked were monitored for 10-30 minutes to ensure that a stable response was obtained. Tetanic stimulation was then delivered as two 100-pulse trains at 100Hz with an inter-train interval of 20 seconds.

2.8.1. Maximal fEPSP size determination

The maximal fEPSP size was determined by increasing the stimulation to the point where a population spike could just be detected (Figure 5, B) and then decreased by a small amount, so that the waveform was smooth and population spike free (Figure 5, A). This method of maximal fEPSP size determination applies to all extracellular experiments performed. Long-term potentiation experiments were performed at one of two stimulation intensities, 25% of the maximal fEPSP slope and 75% of the maximal fEPSP slope. This stimulation level was set at the beginning of each experiment and remained unchanged throughout the recordings. Both $fyn^{-/-}$ and $fyn^{129/Sv-/-}$ experiments (and relevant controls) were performed at both stimulation intensities. $Fyn^{-/-}$ / $FAK^{+/-}$ experiments were performed at the 75% stimulation level. Ras-GRF experiments were performed at the 25% level of stimulation.

2.9. Paired pulse facilitation (PPF) protocol

2.9.1. Extracellular

Synaptic potentials were evoked at 0.2Hz, and the stimulation intensity was set to 50% of the maximal fEPSP slope for the first response. Extracellular PPF recordings were generated by producing two stimulation pulses separated by a short inter-stimulus interval (ISI) of 25, 50, 75, 100, 150, 200 and 300 milliseconds (ms). The initial slope of the fEPSP was used as a measure of synaptic efficacy. Twelve or ten

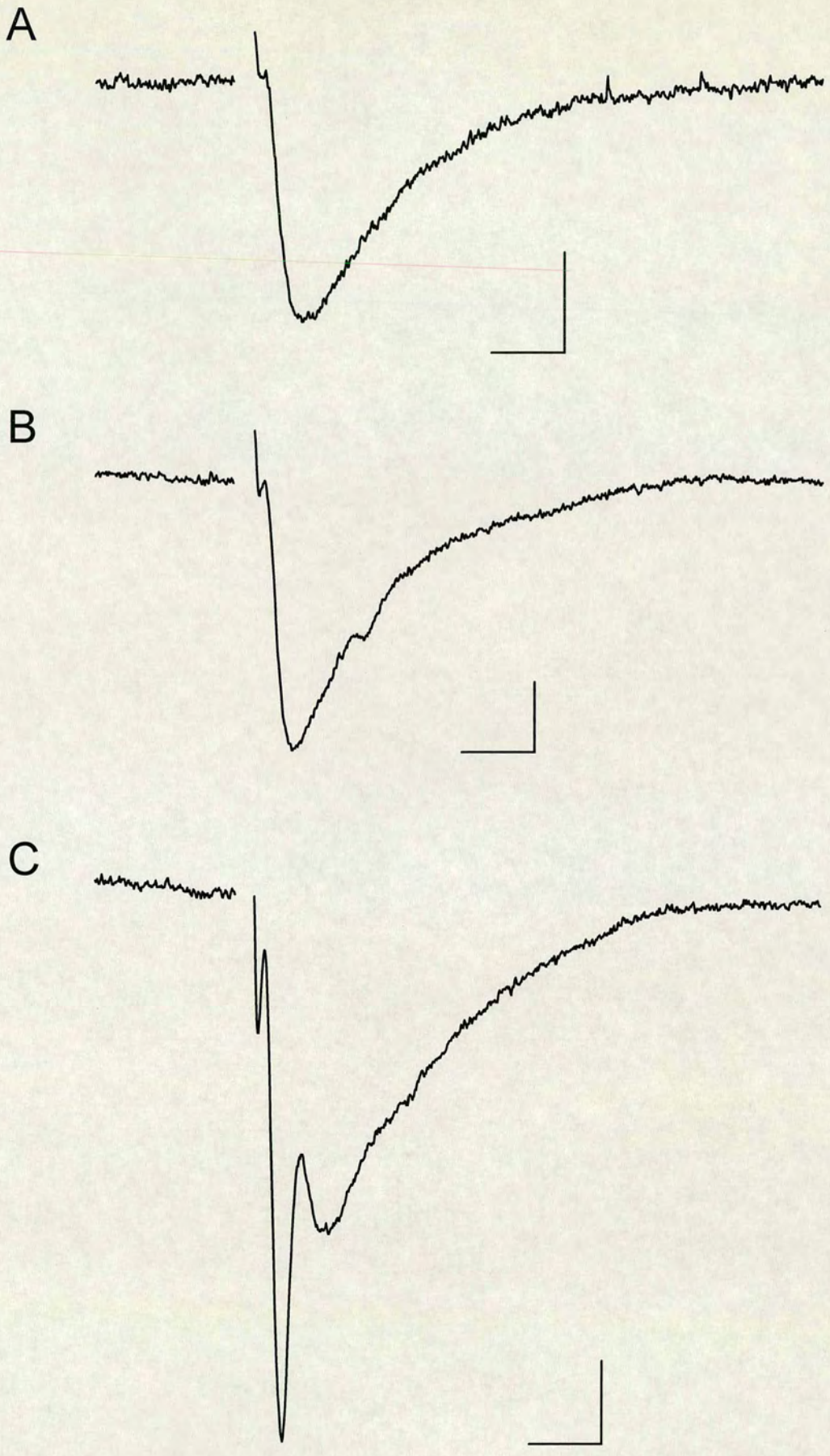
Figure 5 **Determination of maximum fEPSP size**

A Maximum fEPSP as used in this study, the field is smooth and does not include a population spike component.

B Super maximal fEPSP displaying a noticeable inflection during the late phase, indicating a population spike component.

C Grant et al., 1992 method of determining the maximal fEPSP size. Note the large population spike component, with a fast rising phase, contaminating the initial AMPA receptor mediated slope.

Scale bars 0.5mV, 5ms for each graph.


A
B
C

individual sweeps were recorded at each inter-stimulus interval, and the slope measurements were recorded and averaged off line.

2.9.2. Intracellular

AMPAR mediated EPSPs ($EPSP_A$) were isolated by blocking the NMDA, $GABA_A$ and $GABA_B$ receptor mediated components of the synaptic potential with 50μM 40116, 50μM picrotoxin and 1μM CGP55845 respectively applied by bath perfusion. $EPSP_A$s were elicited by stimulation of the Schaffer collateral pathway at 0.1Hz. The stimulation intensity applied (whilst holding the membrane potential at –60mV by injecting current up to a maximum of 0.4nA in bridge mode) was increased until the $EPSP_A$ was just sub-threshold for action potential generation. The stimulus intensity was then set to 50% of the maximal $EPSP_A$ slope for the first response. Paired pulse facilitation was determined at inter-stimulus intervals of 50, 75, 100, 150, 200 and 300ms. The initial slope of the $EPSP_A$ was measured as 25 – 75% of the time to reach peak amplitude. Ten individual sweeps were recorded for each inter-stimulus interval, averaged and the slopes measured off line.

2.9.3. Extracellular calcium manipulations

The PPF profile was determined in 'standard' ACSF for hippocampal slices from $fyn^{129/Sv-/-}$ and wild type mice. The ACSF was changed to a low calcium containing ACSF (1.3mM Ca^{2+}, see Table 1), and perfused for one hour. Paired pulse facilitation data were recorded in the same manner. A high calcium containing solution was then applied (4.5mM, see Table 1). The slices were allowed to equilibrate for one hour before recordings commenced. The profile of paired pulse facilitation was examined in the same manner as described above. Stimulus intensity was maintained at the 50% level set in the 'standard' ACSF for all high calcium experiments. However in certain low calcium experiments the EPSP size reduced to a near zero level (or so small that variability in the slope measurements was very

high). In order to collect data, the stimulus intensity was increased for the duration of the low calcium experiment, and returned to the control level thereafter. Evidence is presented in Figure 5a to demonstrate that PPF ratio is independent of stimulus strength. It should be noted that at very low stimulus intensities, where the conditioning (first) response is very small, the test response (second) is much larger, therefore producing a larger ratio value. In the region of 25 % to maximal fEPSP slope size however, the author finds that the PPF ratio does not alter with increasing stimulus strength. However, another report has detailed a connection between the level of facilitation seen in PPF with stimulus strength and the changes associated with age (see Dumas and Foster, 1995).

2.9.4. Drug applications

Drugs (2-Chloroadenosine (CADO), 8-cyclopentyl1-3,7-dihydro-1,3-dipropyl -1H-purine-2,6-dione (DPCPX), carbachol, atropine, 4-amino-7-phenylpyrazolo [3,4-*d*] pyrimidine (PP3) and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)- pyrazolo [3,4-*d*] pyrimidine (PP2)) were added to the extracellular solution from stock solutions and perfused at their final concentrations for at least fifteen minutes to allow the compounds to equilibrate in the slice tissue. Paired pulse facilitation experiments in which drugs were applied were performed on mice from the 129/Sv inbred background.

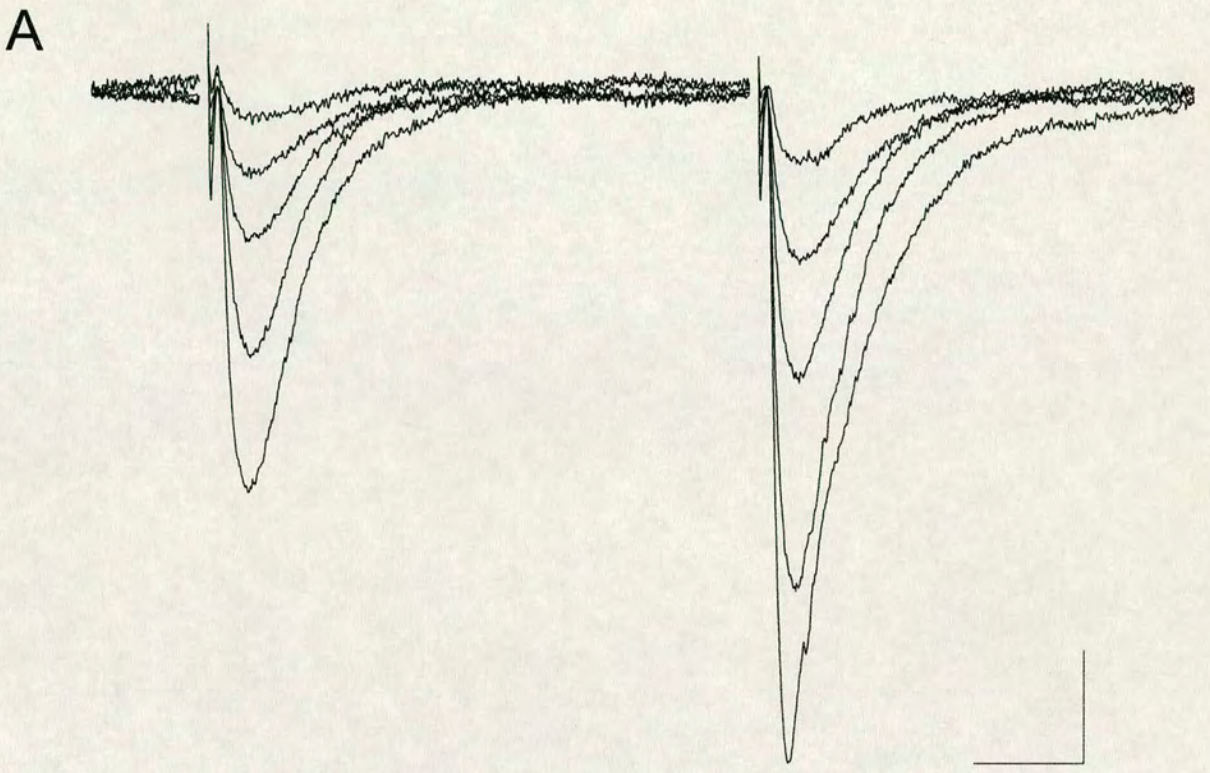
2.10. Input-output (I/O) relationships

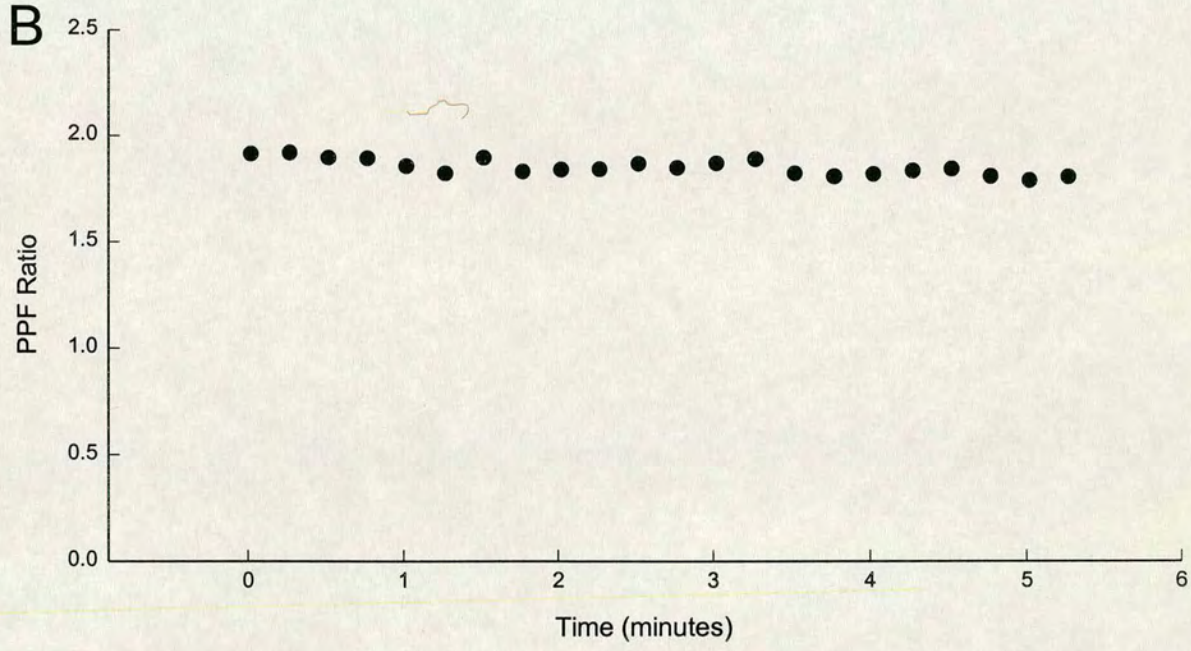
Synaptic responses were elicited at 0.2Hz. The stimulus intensity was initially set to zero and steadily increased in uniform steps. No specific units of stimulus intensity output were measured (that is the amount of current output from the stimulator box), instead the potentiometer dial of an isolated stimulus generator (Iso-Flex, A.M.P.I., Israel) was used to determine the stimulus intensity value. In the case of the hybrid C57BL6 x 129/Sv mice, the I/O relationship was determined at unitary steps of

Figure 5a **Paired pulse facilitation (PPF) and stimulus strength**

A Example trace of PPF during a series of increases in stimulus strength

B Ratio of PPF during the increase in stimulus strength


A


B
PPF Ratio
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
Time (minutes)

stimulus intensity. In the case of the 129/Sv mice, the I/O relationship was determined at 0.25 steps of stimulus intensity. Single fEPSP slopes were recorded until the fEPSP no longer increased in slope or amplitude with increasing stimulus intensity.

2.11. Data analysis

2.11.1. LTP experiments

For each evoked fEPSP the initial slope was measured on line. Each point was normalised to the control (baseline) period by dividing the individual slopes by the averaged response over the 10 minutes prior to tetanisation. Data from each slice was then pooled, off line using the Sigmaplot software package (Ver 3.02, Jandel Scientific, U.S.A.). Figures for potentiation are expressed as the mean ± standard error of the mean (S.E.M.). LTP values are taken as the point 60 minutes post tetanus, STP values are taken at 10 minutes post-tetanus and PTP values are taken from the peak averaged point after tetanisation. LTP, STP and PTP values are quoted as the percentage of potentiation seen from the normalised (100%) control period. Statistical significance between control and experimental data were performed using unpaired t-tests and any effect was considered significant if $P < 0.05$ (95% confidence limit) using the InStat software package (Ver 3.00, Graphpad Software, U.S.A.). The author is aware that the use of repeated t-test on the same time series has a number of limitation regards the validity of results when the number of tests on a single time series increases. This is due to the fact that in a single t-test, at for example the 5 % limit, there is a one in twenty chance that the results obtained could be due to chance alone. As the number of sample groups increases, say to four groups for example (A, B, C & D) the multiple comparisons which have to be made increases to six t-tests (A-B, A-C, A-D, B-C, B-D, C-D). As the number of sample groups increases the number of inter-sample comparisons increases at a much faster rate. Thus, six samples would involve 15 tests, eight would involve 28, ten would involve 45, and so on. The greater the number of separate test made, the greater the

likelihood that some differences will be claimed as real when they are actually due to chance.

In the case of the LTP experiments presented in this thesis, the number of sample groups is small (three, i.e. LTP, STP & PTP) therefore the problem is not particularly significant. Biologically speaking the most important result for this thesis is the LTP result, which can be counted as the most important t-test in this series, so little weight should be given to the statistical results from the PTP and STP figures, although visual inspection of the averaged data sets (see graphs) allows use to make a firmer assumption that in all cases the PTP and STP are the same between all groups.

2.11.2. PPF experiments

The ratio of the initial slope of the second response (S_2) to the initial slope of the first response (S_1) was used to express the level of facilitation seen (S_2 / S_1). Either twelve or ten successive synaptic responses (pairs of fEPSP / IPSPSs) were recorded at each inter-stimulus interval. For each inter-stimulus interval successive fEPSP slopes were averaged. The ratio of facilitation at all intervals for each slice was then pooled for each series of experiments using Sigmaplot 3. Points are expressed as the mean ± S.E.M.. Statistical significance comparisons were performed using one way analysis of variance (ANOVA) test and any effect was considered significant if the P value was less than 0.05 (95% confidence limit, InStat software). The use of the one way ANOVA test in these cases are justified upon the basis of the biological meaning given to the data. The real question that is asked in all PPF experiments (in effect our null hypothesis) is "Is there an effect of the genetic ablation of fyn tyrosine kinase when compared to wild type controls". That is the statistical test is used to determine if there is or is not a difference based on genotype, not based on drug treatments, interval or any other manipulation (e.g. age). In order to make these comparisons the use of multiple factor ANOVAs is required.

2.11.3. Input / Output relationship

In the case of the hybrid C57BL6 x 129/Sv mice, the fEPSP slope and the fibre volley amplitude of a single synaptic potential was measured. Fibre volley amplitudes were both automatically and manually calculated using the Mintra software (Hand written LTP software, © David Selig). The individual fEPSP slopes were normalised against the maximal fEPSP slope (as determined above) and pooled between slices. In the case of 129/Sv mice, fEPSP slope alone was measured. The fEPSP slope was normalised against the maximal fEPSP size (as above) and pooled between slices. Averaged data sets are plotted as mean normalised fEPSP slope ± S.E.M. versus stimulus intensity (arbitrary units) using Sigmaplot 3. Statistical significance was tested using a students t-test on the maximal stimulus intensity. If there was to be any difference in the group means, then it is at this level where the most significant effect would be seen, thus only this point was chosen for statistical analysis, to avoid the problem of multiple t-tests on the same sample group (see above).

2.12. Selection criteria

Selection criteria were applied to hippocampal slices to ensure that recordings were made from optimal preparations. These are detailed below:

2.12.1. Hippocampal slice criteria

1. Fibre volley amplitude less than one third fEPSP amplitude.
2. No signs of compromised inhibition (multiple population spikes).
3. Maximum field EPSP size: Submerged chamber 1mV or greater
   Interface chamber 4mV or greater
4. Stable response over time.

2.12.2. Hippocampal CA1 pyramidal cell criteria

1. Action potentials cross 0mV in all cases.
2. The holding current to maintain membrane potential at –60mV in bridge mode was less than -0.4nA.

2.13. Drugs

Drugs were stored frozen in stock aliquots (50µl – 5ml) of 100 to 10000 times final concentration. Drugs were dissolved in either ACSF or DMSO and administered by bath perfusion. All experiments using drugs were performed with adequate vehicle controls. They were obtained from the following sources:

CGP55485	Tocris Neuramin
40116	Tocris Neuramin
Carbachol	Tocris Neuramin
2-Chloroadenosine (CADO)	Sigma
Picrotoxin	Sigma
4-amino-7-phenylpyrazolo [3,4-*d*] pyrimidine (PP3)	Calbiochem
4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo [3,4-*d*] pyrimidine (PP2)	Calbiochem
8-cyclopentyl1-3,7-dihydro-1,3-dipropyl -1H-purine-2,6-dione (DPCPX)	Tocris Neuramin

2.14. Anatomical Procedures

2.14.1. Tissue Sectioning

Mice were decapitated under halothane anaesthesia, and all overlying cranial tissue removed. Whole brains were removed from the cranial cavity in an intact state by

carefully removing and cutting the cranial bones. Intact brains were frozen by burial in powered dry ice, and stored at -70°C until required. Brains were frozen embedded in 'tissue-tek' on the freezing block of a cryostat. Sections (20-50μm) were taken throughout the hippocampus and in certain cases from the whole brain, and thaw mounted onto 'tespa' (3-aminopropyltriethoxysilane) coated slides. These were stored at -70°C until stained with cresyl violet (see below) for gross morphological examination.

2.14.2. Staining Protocol - Cresyl Violet

Slides were immersed for 5 minutes in each of the following: Xylene, xylene, 100% alcohol, 100% alcohol, 95% alcohol and 70% alcohol. Sections were then dipped in distilled water (dH_20) and stained in a 0.5% cresyl violet solution for 15-30 minutes. To differentiate the staining, sections were immersed in dH_20 for 0.5 - 2 hours whilst checking the level of staining under a dissection microscope. Slides were then de-hydrated through 70%, 95%, 100% and 100% alcohol (Paxinos and Watson, 1986). Slides were washed with 100 % xylene and cover slipped using DPX (BDH Chemicals). Photographic images were taken through a binocular microscope under low power magnification.

2.15. Genotyping of Mice

The genotype of mice was established using the polymerase chain reaction (PCR) technique. Specific fyn primers were designed by, and a kind gift from, Dr Philippe Soriano. One primer (Neo F4) recognises an internal site in the neomycin-targeting construct. The other two primers (Fyn i2R3, Fyn i2F3) recognise forward and reverse sites of the Fyn gene loci.

The three primers have the following nucleotide sequence:

Fyn i2R3	(5'-TACTCCCAACGCTCACTA)
Fyn i2F3	(5'-GTCCCTCTTCCCACTCTTC)
Neo F4	(5'-CGCCTTCTATCGCCTTCTT)

The PCR reaction amplified a single band of 450 base pairs (bp) for homozygous animals, 270bp for the wild type animals and for heterozygotes both bands (450 and 270bp) were amplified. The protocol for this procedure is detailed below. An example photograph of a genotyping gel is shown in Figure 6.

2.15.1. Tail Digest

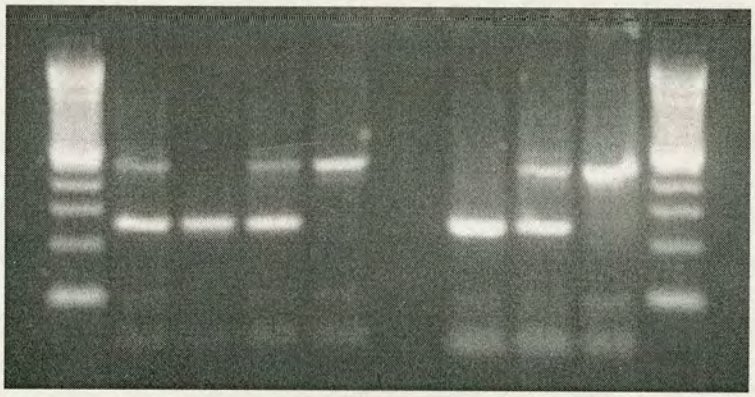
Tail samples were taken from pups at 4 weeks of age. Samples of tail (1cm) were placed in 1.5ml Eppendorf tubes and digested in a tail buffer solution consisting of: 0.3M Sodium Acetate, 10mM Tris-HCL (pH 8.5), 1 mM EDTA, 1% SDS and 200 μg/ml proteinase K. Tubes were shaken at 55°C overnight and then left at 4°C for 24 hours.

2.15.2. PCR reaction

Individual PCR tubes for each tail sample were prepared on ice containing:

5μl Buffer (100mM Tris-HCl (pH 9.0), 500mM KCl, 1% Triton X-100)
3μl 25mM $MgCl_2$
2μl dNTPs (200μM dCTP, 200μM dATP, 200μM dGTP, 200μM dTTP)
~ 40pM Fyn i2R3 primer
~ 40pM Fyn i2F3 primer
~ 40pM Neo F4 primer
dH_2O to 50μl

Two drops of mineral oil were placed on top of this mixture (to avoid evaporation) and the tubes were sterilised using a strong ultra violet light for 3 minutes. Tail lysates were spun at 3000rpm at 4°C for 15 minutes. 2µl aliquots of the uppermost layer were added through the oil layer, giving a final volume of 50µl. A hot start PCR protocol was used. The tubes were heated to 94°C and left for 15 minutes to promote dissociation of the genomic DNA. 0.5 to 1µl of Taq DNA polymerase (5 units / µl, Promega, U.S.A.) enzyme was added to each tube. 40 cycles of PCR (93°C for 1 minute, 51°C for 1 minute, 65°C for 1 minute) were performed on a Hybaid Omigene PCR temperature cycling machine. Samples were then electrophoretically separated on a 2% agarose gel containing ethidium bromide, at 70V for 1.5 hours. Bands were visualised and photographed under UV light, and the relevant genotype established against control samples and a 1kB-marker ladder (Promega, U.S.A.).



Lanes 1 2 3 4 5 6 7 8 9 10

Figure 6 – Example genotyping gel

Lanes 1 and 10 show the 1kB ladder. The first band is 100bp, and increases in 100bp steps. Lanes 7, 8 and 9 show control wild type (+/+), heterozygote (+/-) and homozygote (-/-) bands respectively. Lane 6 is the blank control lane. Lanes 2, 3, 4 and 5 show experimental tail digest, indicating heterozygous (+/-), wild type (+/+), heterozygous (+/-) and homozygous (-/-) animals in this group.

2.16. Summary of animal strains, bath types and experiments performed

5 groups of animals from differing genetic backgrounds were used in the experiments presented in this thesis. These are as follows:

$Fyn^{-/-}$ animals	(Hybrid C57BL6 x 129/Sv strain)
$Fyn^{-/-}$ / $FAK^{+/-}$ animals	(Hybrid C57BL6 x 129/Sv strain)
Wild type hybrid animals	(Hybrid C57BL6 x 129/Sv strain)
$Fyn^{129/Sv-/-}$ animals	(Pure 129/Sv inbred strain)
Wild type 129/Sv animals	(Pure 129/Sv inbred strain)
Ras-GRF mutants	(C57BL6 strain)

Table 2 - Summary of experiments performed

Animal strain	***Experiment***	***Chamber type***	***Figure number***
$Fyn^{-/-}$	LTP at 75%	Submerged	9
Wild type hybrid	LTP at 25%	Submerged	10
	LTP at 25%	Interface	11
	PPF profile	Submerged	19
	I/O relationship	Submerged	14
$Fyn^{129/Sv-/-}$	LTP at 75%	Submerged	15
Wild type 129/Sv	LTP at 25%	Submerged	16
	PPF profile	Submerged	20
	I/O relationship	Submerged	14
	PPF PP2 / PP3	Submerged	21
	PPF Age	Submerged	22
	PPF Intracellular	Submerged	23
	PPF Calcium	Submerged	24, 25
	PPF Carbachol	Submerged	26
	PPF CADO	Submerged	27
$Fyn^{-/-}$ / $FAK^{+/-}$	LTP at 75%	Submerged	8
Ras-GRF	LTP at 25%	Submerged	7

Chapter III

Results

Long-term potentiation, morphology and basal synaptic transmission

3.1. Ras-GRF long-term potentiation experiments

Ras-GRF is a molecule involved in the $p21^{ras}$ signalling cascade that interacts with the src family of tyrosine kinases in a calcium dependent manner. LTP experiments were performed at the 25% level of maximal fEPSP slope stimulus intensity in Ras-GRF knockout and littermate controls (Figure 7). The averaged level of potentiation at sixty minutes post-tetanus was not significantly different for Ras-GRF knockout mice (163 ± 14 %, n = 11) compared to wild type mice (159 ± 14 %, n = 13). Equivalent levels of both PTP (310 ± 55 % Ras-GRF mice, 409 ± 41 % wild type mice) and STP (191 ± 19 % Ras-GRF mice, 190 ± 8 % wild type mice) were seen. No significant differences were noted at the PTP, STP and LTP time points (P = 0.2, P = 1.0 and P = 0.8 respectively).

3.2. $Fyn^{-/-}$ / $FAK^{+/-}$ long-term potentiation experiments

To determine if FAK is involved in a Fyn dependent signalling pathway regulating LTP in area CA1 of the hippocampus, LTP was studied in mice homozygous for the deletion of fyn and heterozygous for the deletion of FAK ($fyn^{-/-}$ / $FAK^{+/-}$). A stimulus level equivalent to 75% of the maximal fEPSP slope was used to induce LTP, as the FAK mutation was predicted to reduce the level of potentiation further than that seen in the $fyn^{-/-}$ mice. An example of an individual experiment is shown in Figures 8A and 8B. Pooled data from $fyn^{-/-}$ / $FAK^{+/-}$ mice showed a similar level of LTP at sixty minutes post tetanus (151 ± 9 %, n = 8) as seen in wild type mice (155 ± 8 %, n = 15, Figure 8C). There was a very small trend towards a lower level of potentiation in the initial part of the experiment in $fyn^{-/-}$ / $FAK^{+/-}$ mice, however this did not achieve statistical significance at the PTP, STP or LTP time points (P = 0.8, P = 0.2, P = 0.8 respectively).

Figure 7 **Ras-GRF long-term potentiation experiment (See 3.1)**

A An individual example of an LTP experiments in the Ras-GRF mice. Tetanus was applied at the ten minute time point, producing a significant and maintained increase in fEPSP size and initial slope. Inset, example traces of responses pre and post tetanus, scale bar 0.25mV, 5ms.

B As above, for wild type control animals.

C Pooled data for Ras-GRF (white squares, n = 11 slices) and wild type control (black circles, n = 13 slices) animals at the 25% level of stimulation. The slope of the field EPSP was normalised to the averaged control value for each experiment and then results from each experiment were averaged. The error bars indicate the standard error of the mean.

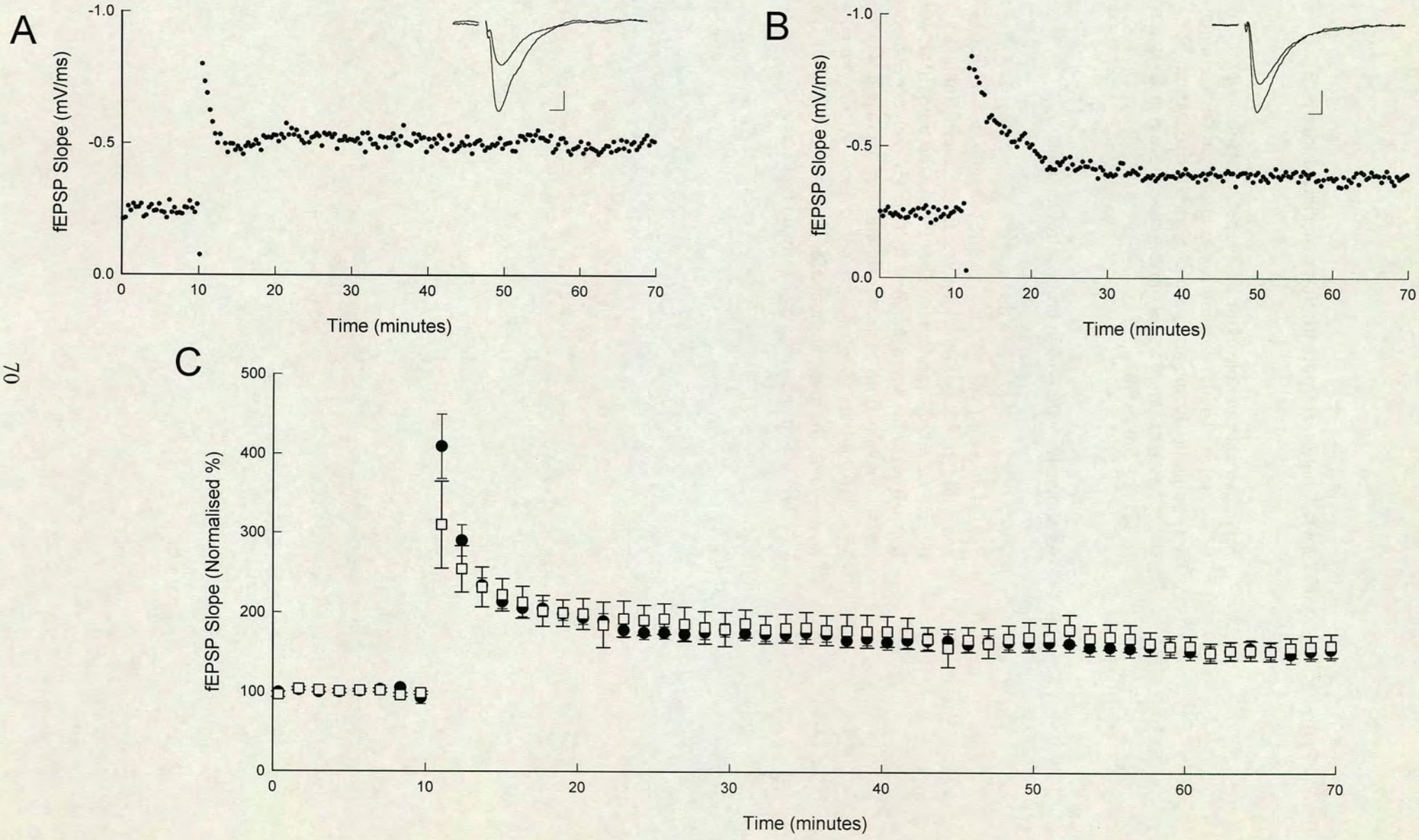
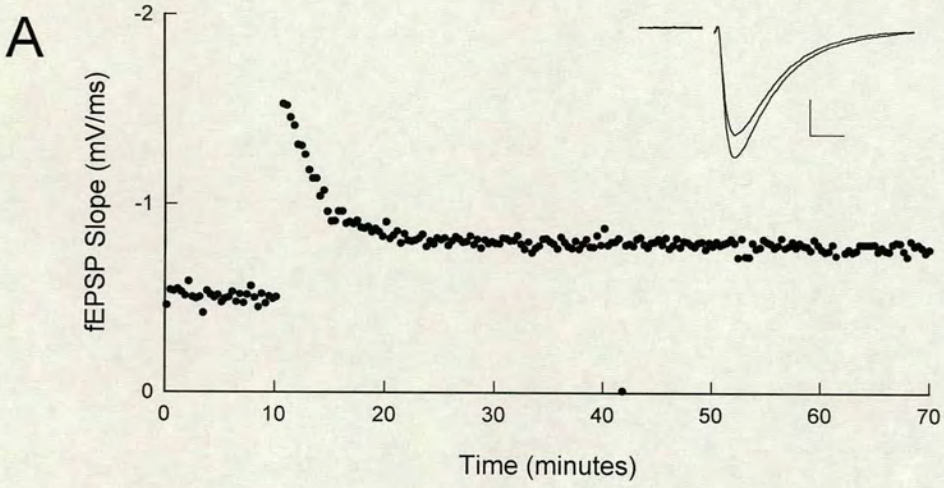

A
-1.0
-0.5
0.0
fEPSP Slope (mV/ms)
0
10
20
30
40
50
60
70
Time (minutes)
B
-1.0
-0.5
0.0
fEPSP Slope (mV/ms)
0
10
20
30
40
50
60
70
Time (minutes)
C
500
400
300
200
100
0
fEPSP Slope (Normalised %)
0
10
20
30
40
50
60
70
Time (minutes)

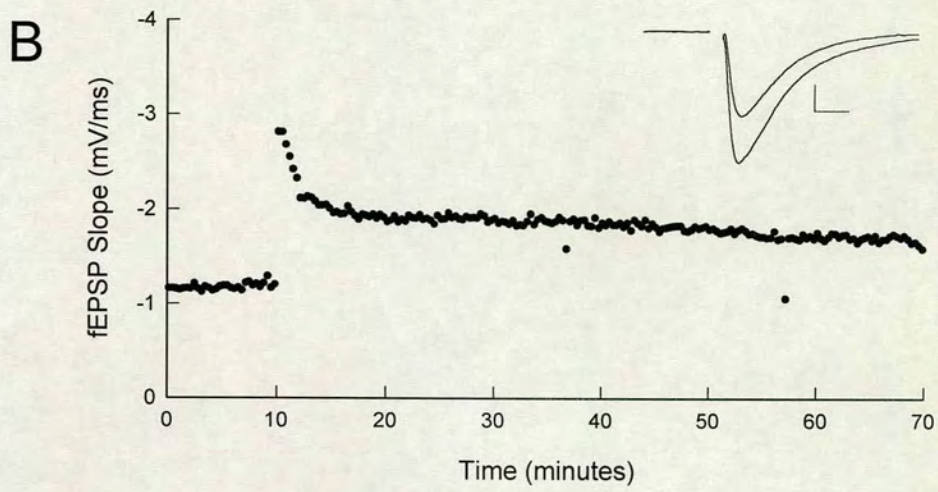
Figure 8 **$Fyn^{-/-}$ / $FAK^{+/-}$ long-term potentiation experiment (See 3.2)**

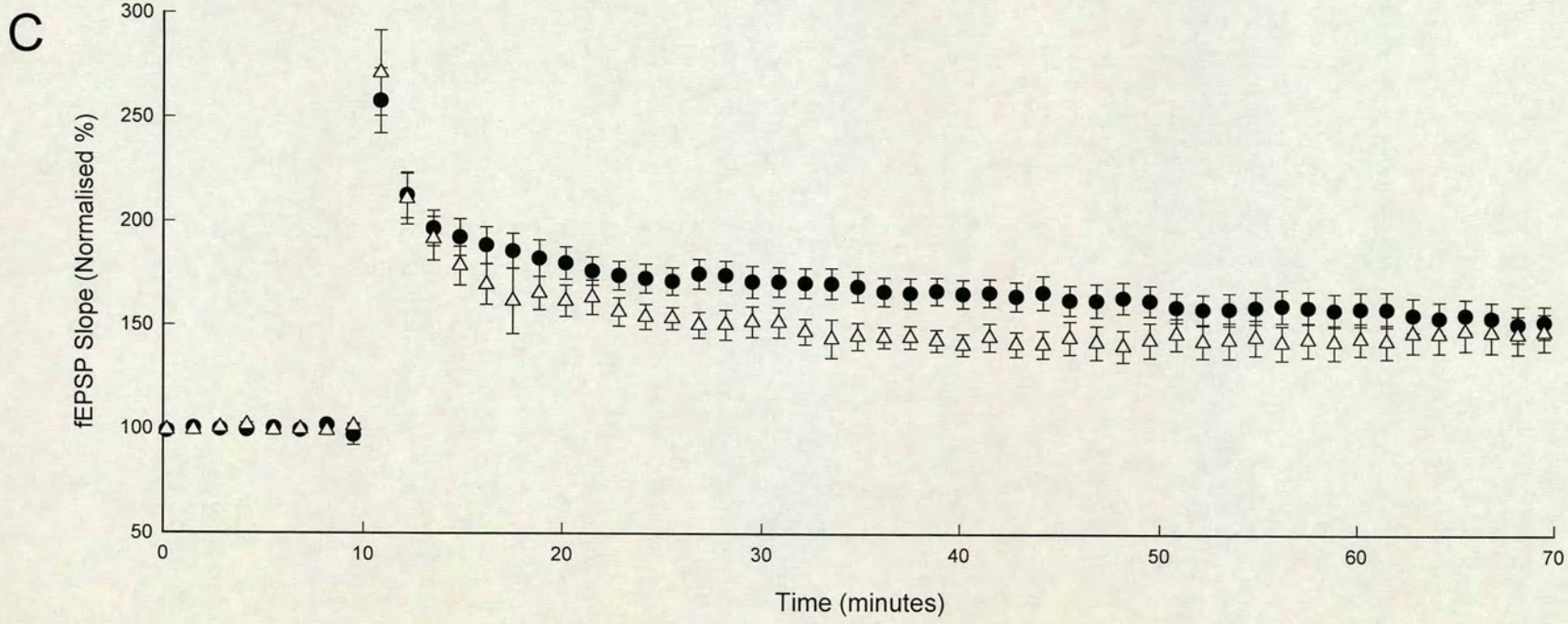
A An individual example of an LTP experiments in the $Fyn^{-/-}$ / $FAK^{+/-}$ mice. The LTP stimulus was applied at the ten minute time point, producing an increase in fEPSP size and initial slope. Inset, example traces of responses pre and post tetanus, scale bar 0.5mV, 5ms.

B As above, for wild type hybrid control animals.

C Pooled data for $Fyn^{-/-}$ / $FAK^{+/-}$ (white triangles, n = 8 slices) and wild type control (black circles, n = 15 slices) animals at the 75% level of stimulation. The slope of the field EPSP was normalised to the averaged control value for each experiment and then results from each experiment were averaged. The error bars indicate the mean ± standard error. At sixty minutes post tetanus, the mean amount of LTP was: 151 ± 9 % for $fyn^{-/-}$ / $FAK^{+/-}$ mice and 155 ± 8 % for wild type mice.


A
fEPSP Slope (mV/ms)
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)


B
fEPSP Slope (mV/ms)
-4
-3
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)


C
fEPSP Slope (Normalised %)
300
250
200
150
100
50
0
10
20
30
40
50
60
70
Time (minutes)

3.3. $Fyn^{-/-}$ long-term potentiation experiments

Due to the lack of effect in the $fyn^{-/-}$ / $FAK^{+/-}$ mice, experiments were performed to determine the level of potentiation seen in mice lacking only Fyn tyrosine kinase ($fyn^{-/-}$), which has previously been reported to be significantly impaired (Grant et al., 1992). The experimental protocol was identical to that used for the $fyn^{-/-}$ / $FAK^{+/-}$ mice (i.e. 75% maximal fEPSP slope level of stimulation). Individual and averaged results are shown in Figure 9. No significant difference was seen in the levels of LTP between the two groups at sixty minutes post-tetanus, $fyn^{-/-}$ (143 ± 8 %, n = 9) and wild type (155 ± 8 %, n = 15, P = 0.4). PTP and STP were also not significantly different (P = 0.8, P = 0.2 respectively).

The LTP impairment seen in the $fyn^{-/-}$ mice is not absolute, and has been reported to be stimulation level dependent (Grant et al., 1992). To determine if the 75% stimulus level used in the two studies ($fyn^{-/-}$ & $fyn^{-/-}$ / $FAK^{+/-}$) was masking the reduction of LTP in these mice, the experiment was repeated using the 25% maximum fEPSP stimulation level. Wild type mice showed a similar level of LTP at 60 minutes post-tetanus (160 ± 8 %, n = 10) as compared to the $fyn^{-/-}$ mice (166 ± 13 %, n = 8, not significant, P = 0.8, Figure 10). Both PTP and STP were also found not to be significantly different (P = 0.6, P = 1.0). These LTP results are summarised in Table 3.

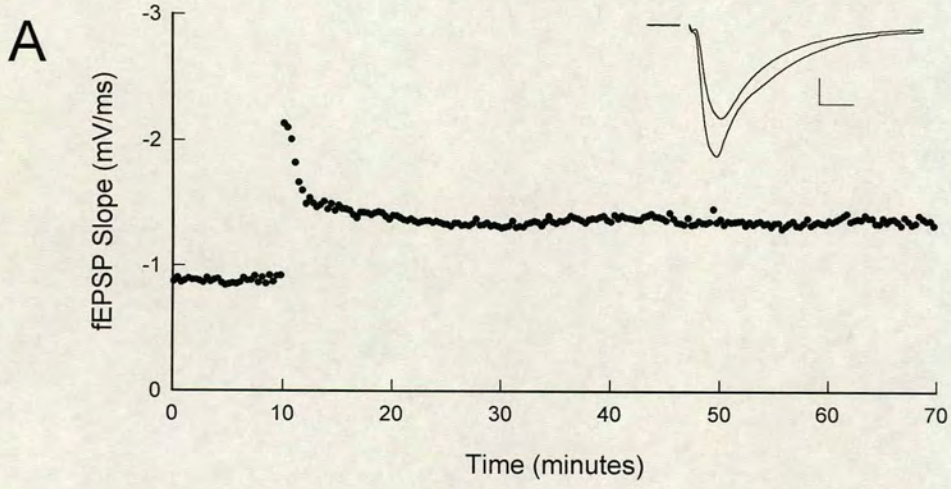
The reason for the apparent lack of impairment in LTP in the $fyn^{-/-}$ mice was not clear. Exact details of the Grant et al. experimental protocol were obtained from the publication and the electrophysiologist involved (Thomas O'Dell, Personal communication) so that the experiments could be repeated identically. Several aspects of the experimental procedure were different between the results presented here and the results from the Grant et al. paper. These differences included the type of recording chamber used, flow rate, temperature, stimulus protocol, slice preparation and storage, ACSF composition, measurement of maximal field EPSP size and age and genetic background of the mice.

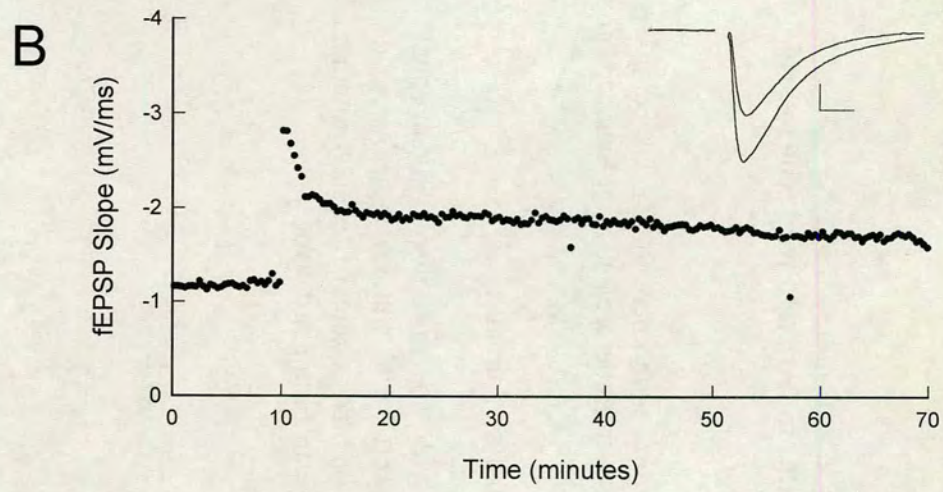
Figure 9 **LTP in $fyn^{-/-}$ and wild type hybrid animals at the 75% level of stimulation. (See 3.3.)**

A Examples of individual experiments in $fyn^{-/-}$. Insets display the pre and post tetanus fEPEPs. Scale bars are 0.5mV, 5 ms.

B As above for wild type hybrid controls

C Each experiment was normalised against its own control period and the averaged data set plotted against time. The points represent the mean LTP ± the standard error. The mean amount of LTP after 60 minutes was: 143 ± 8 % for $fyn^{-/-}$ mice (white triangles) and 155 ± 8 % for wild type hybrid control animals (black circles). Error bars indicate the mean ± standard error.


A
fEPSP Slope (mV/ms)
-3
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)


B
fEPSP Slope (mV/ms)
-4
-3
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)

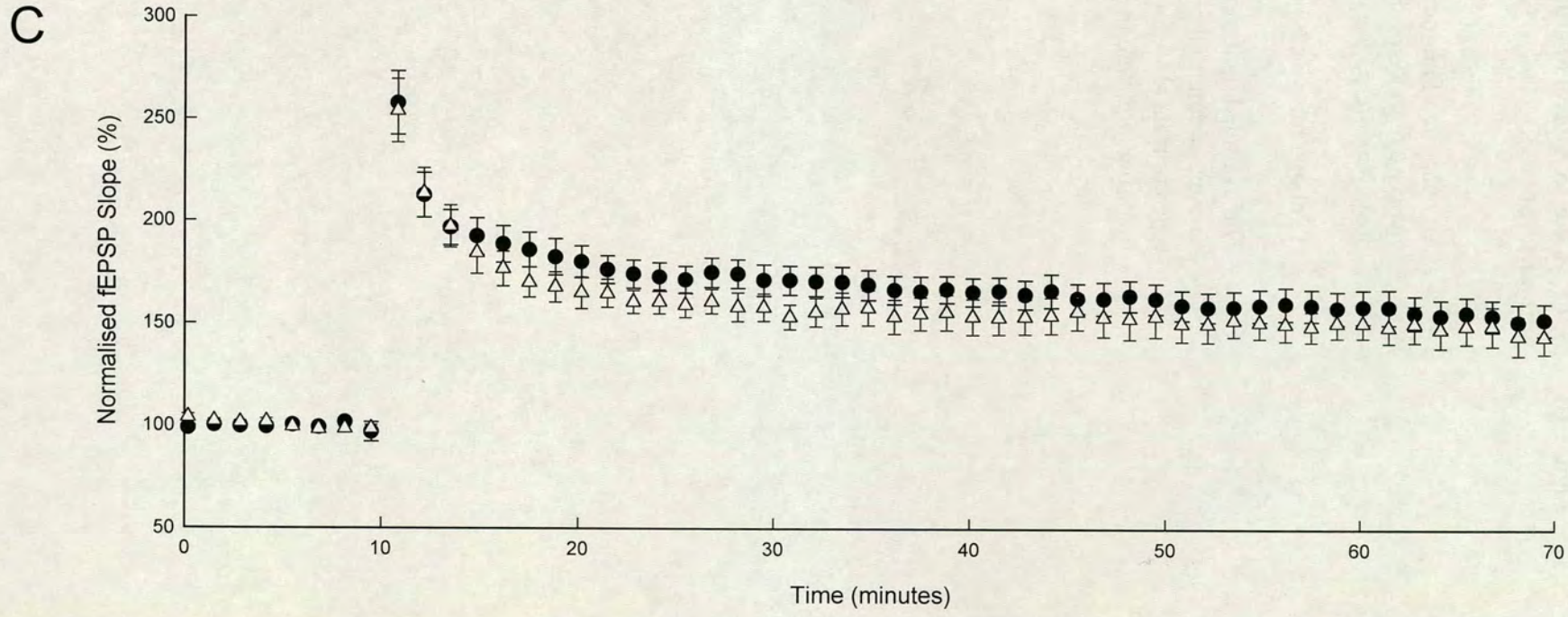
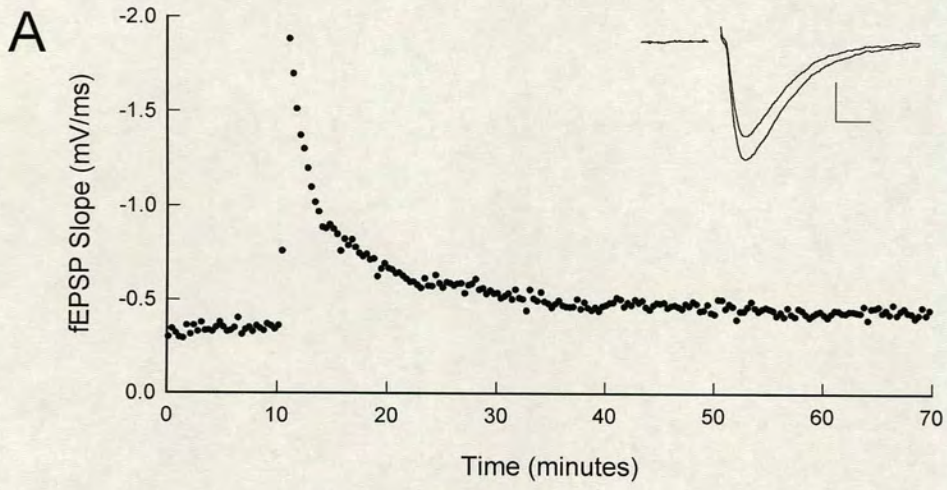

C
Normalised fEPSP Slope (%)
300
250
200
150
100
50
0
10
20
30
40
50
60
70
Time (minutes)

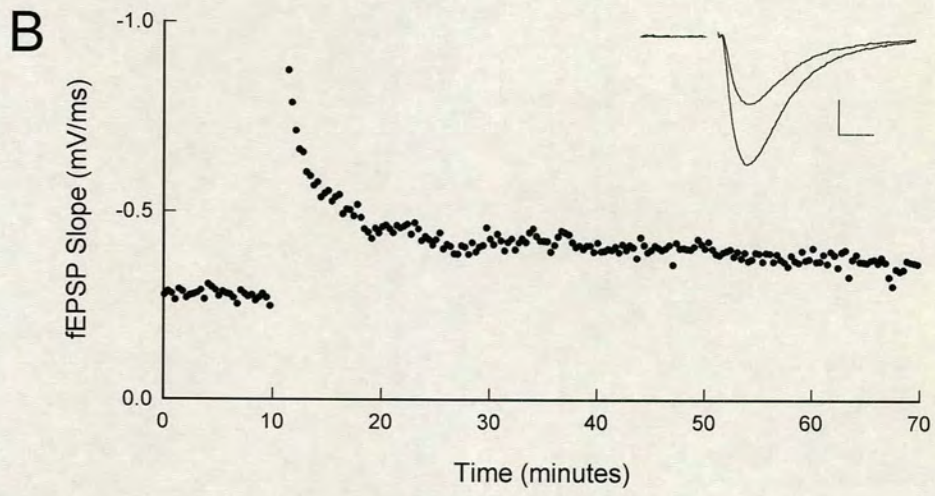
Figure 10 **LTP at the 25% level of stimulus in $fyn^{-/-}$ and wild type hybrid animals. (See 3.3.)**

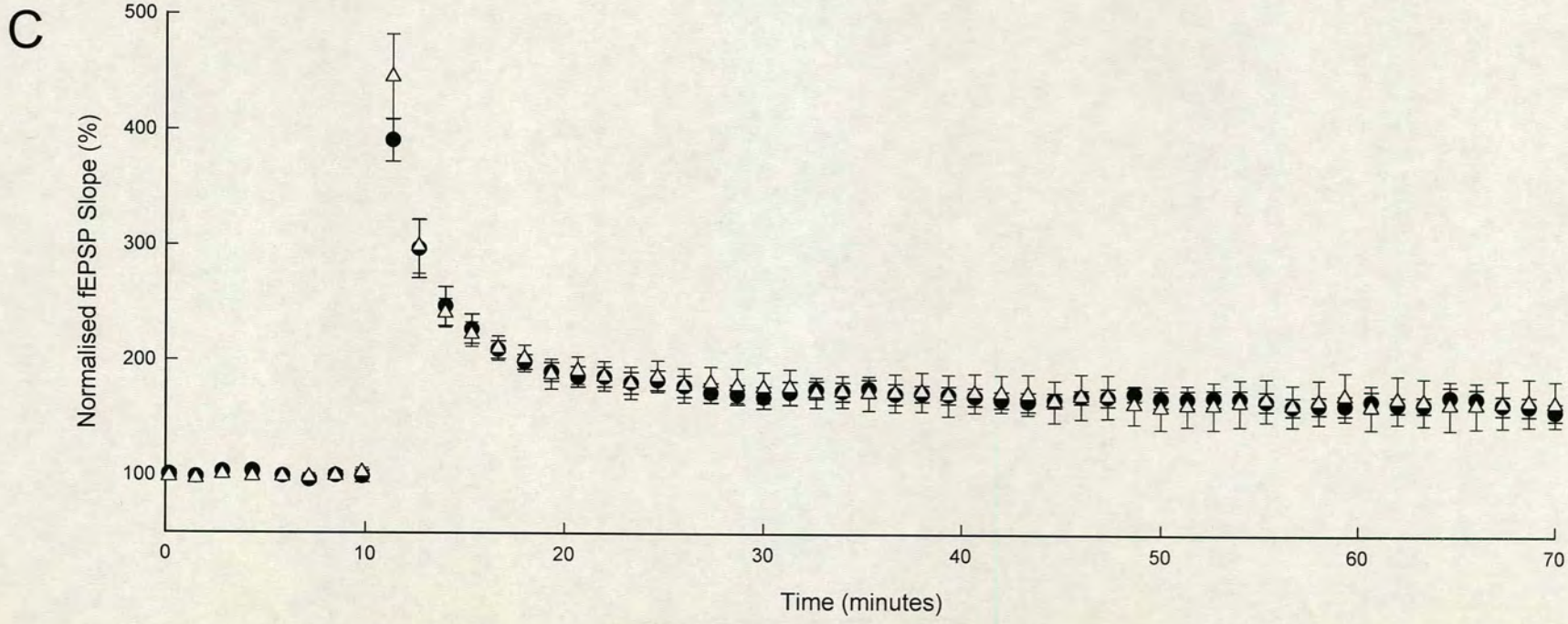
A An example of an individual experiment for $fyn^{-/-}$ mice. Insets display examples of traces from before and after tetanisation. Scale bars are 0.25mV, 5ms.

B As above for wild type hybrid animals

C The averaged data sets, with $fyn^{-/-}$ mice displaying an equal amount of potentiation (160 ± 8 %, white triangles) as wild type controls (166 ± 13 %, black circles) after sixty minutes. Error bars indicate the mean ± standard error.


A
fEPSP Slope (mV/ms)
-2.0
-1.5
-1.0
-0.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)


B
fEPSP Slope (mV/ms)
-1.0
-0.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)


C
Normalised fEPSP Slope (%)
500
400
300
200
100
0
10
20
30
40
50
60
70
Time (minutes)

The LTP experimental protocol was amended to take all of these parameters into consideration. Details of changes in experimental protocol used to repeat the Grant et al. study are outlined below.

3.3.1. Replication of Grant et al. 1992 fyn$^{-/-}$ long-term potentiation experiment

The results presented so far in this thesis were generated in a submerged type of recording chamber, whereas the Grant et al. experiments were performed in an interface type chamber. In order to determine if this difference had any effect on the phenotype, the experiment was repeated in an interface type of chamber (Dr Ceri Davies, The Department of Pharmacology, University Of Edinburgh). The composition of the ACSF was altered to that used in the Grant et al. study (see Table 1). 50μM, D,L-2-amino-5-phophonovaleric acid (D-AP5) was included in the ACSF used for slice preparation to avoid over-activation of NMDA type receptors and possible calcium induced neuro-toxicity. Exactly the same stimulating electrodes were used to maintain consistency throughout the data sets (i.e. all possible steps were taken to ensure that this set of experiments was performed in the same manner as the Grant et al. study).

Examples of individual experiments and the pooled data generated at the 25% maximum fEPSP level of stimulation using this protocol are presented in Figure 11. These results again showed no difference in the level of LTP obtained for wild type (187 ± 26 %, n = 8) and fyn$^{-/-}$ (166 ± 10 %, n = 6, P = 0.4) mice at sixty minutes post-tetanus. Similarly, no effect of the fyn knockout was seen at the PTP and STP time points (P = 0.7, P = 0.8). No statistical difference was seen in the results generated from the submerged type chamber to those generated in the interface type chamber (P = 0.3 for wild type and P = 1.0 for fyn$^{-/-}$). The composition of the ACSF did not affect the levels of potentiation seen in either group of mice.

This entire series of LTP experiments is summarised in Table 3, along with data from the Grant et al., 1992 paper.

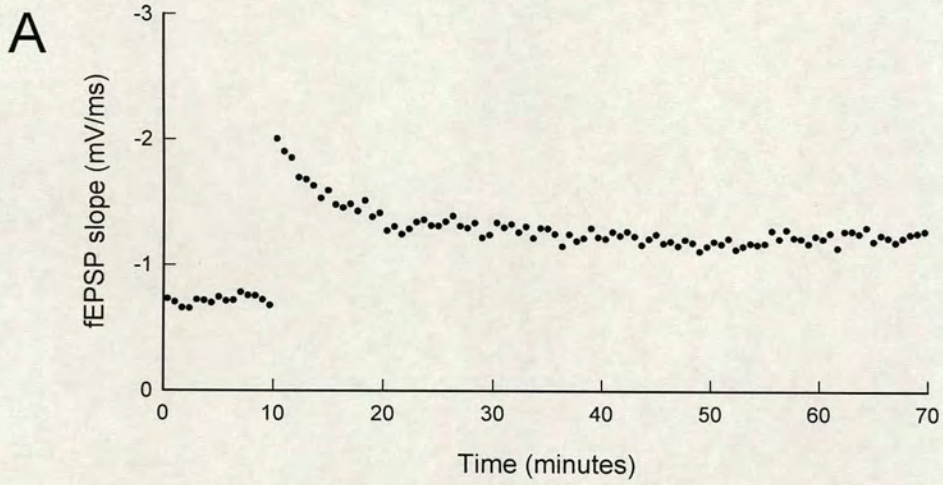
Figure 11 **Replication of the Grant et al., 1992 LTP experiment. (See 3.3.1)**

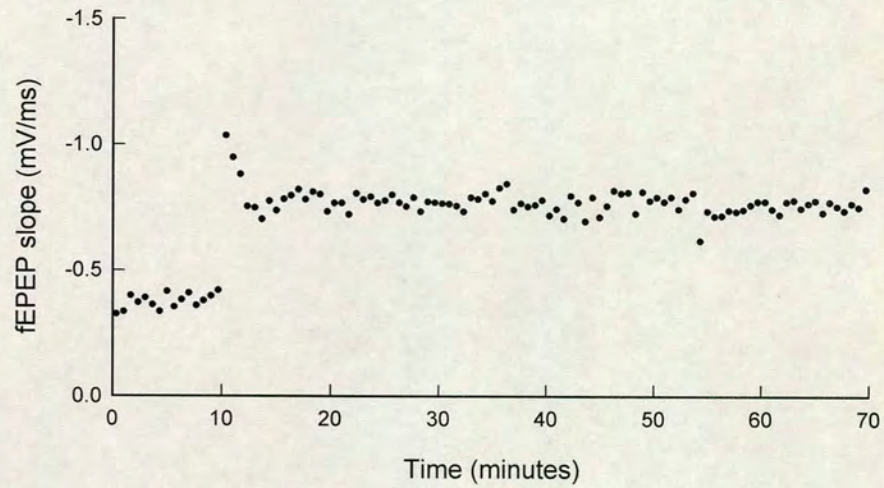
Experimental conditions were aligned to the protocol used in the Grant et al., 1992 study. These experiments were performed in an interface type of chamber.

A Individual example of $fyn^{-/-}$ experiment

B As above for wild type hybrid animals

C The mean amount of potentiation sixty minutes after tetanus was not significantly different in $fyn^{-/-}$ animals (166 ± 10 %, white triangles) compared to wild type animals (187 ± 27 %, black circles). Error bars indicate the mean ± standard error.


A
fEPSP slope (mV/ms)
-3
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)


B
fEPEP slope (mV/ms)
-1.5
-1.0
-0.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)

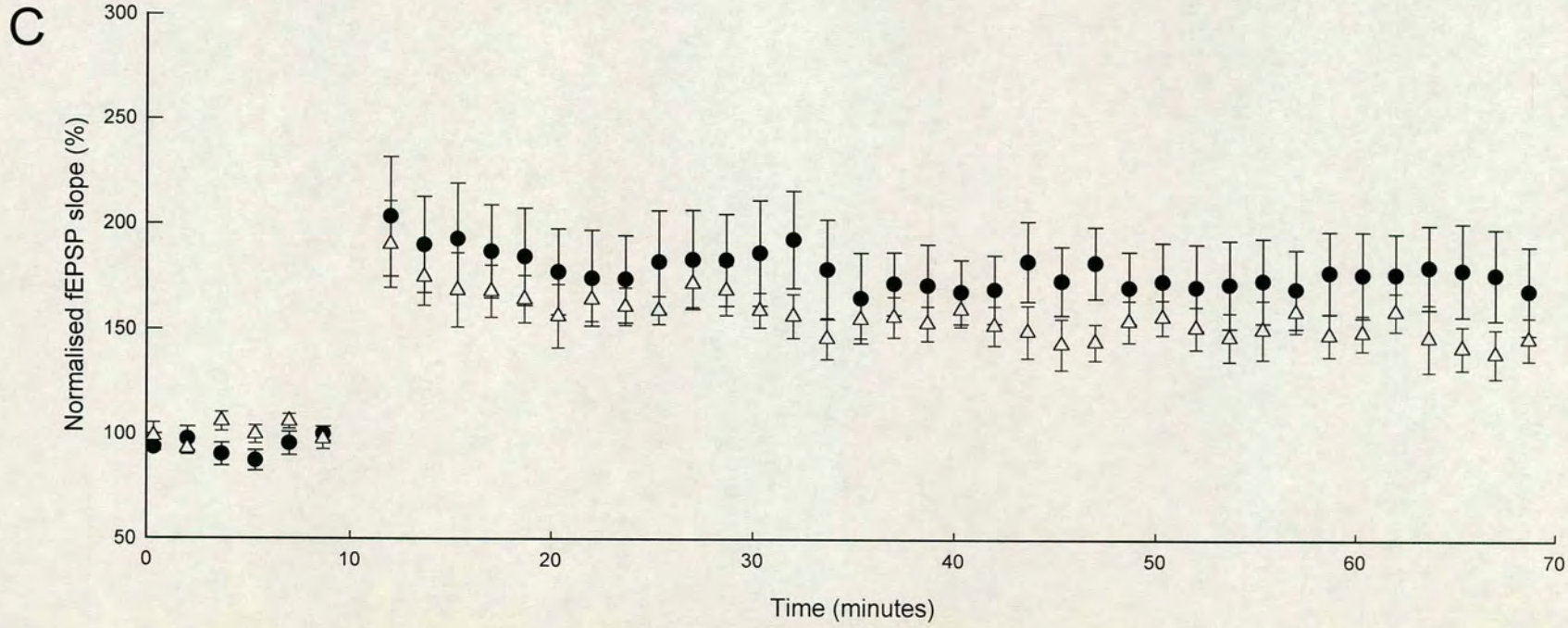

C
Normalised fEPSP slope (%)
300
250
200
150
100
50
0
10
20
30
40
50
60
70
Time (minutes)

Table 3 – Summary of long-term potentiation results in $fyn^{-/-}$ and wild type hybrid animals

Experiment	*LTP ± S.E.M.*	*N*	*Significant*	*P Value*
C57 Wild Type 75% Submerged	154.7 ± 8.3	11	No	0.3752 (Wt, Fyn)
C57 Fyn 75% Submerged	143.3 ± 8.2	6	No	0.5632 (Fyn, FynFAK)
C57 Fyn FAK 75% Submerged	150.6 ± 9.1	4	No	0.7587 (Wt, FynFAK)
C57 Wild Type 25% Submerged	159.9 ± 7.7	10	No	0.7906 (Wt,Fyn)
C57 Fyn 25% Submerged	166.3 ± 12.8	7		
C57 Wild Type 25% Interface	187.4 ± 25.7	8	No	0.4497 (Wt,Fyn)
C57 Fyn 25% Interface	165.5 ± 9.8	6		
Grant et al. Study 1mV Wild Type	177.5 ± 2.9	7	Yes	<0.0001 (Wt,Fyn)
Grant et al. Study 1mV Fyn	90.5 ± 4.5	11		
Grant et al. Study 25% Wild Type	149.6 ± 18.2	9	No	0.0611 (Wt,Fyn)
Grant et al. Study 25% Fyn	108 ± 7.6	8		
Grant et al. Study 75% Wild Type	168.5 ± 11.6	12	Yes	0.0415 (Wt,Fyn)
Grant et al. Study 75% Fyn	133 ± 9.3	8		

Note: No significant difference was seen at the 25% level of stimulation in the Grant et al., 1992 study

3.4. Hippocampal morphology in fyn$^{-/-}$ mice

Gross examination of hippocampal morphology was used to determine if the fyn$^{-/-}$ mice used in this study displayed the characteristic phenotype described by Grant et al.. In the granule cell layer of the anterior dentate gyrus there appeared to be an increase in the cell body layer width, with several examples of distorted morphology (Figure 13). The granule cell layer of anterior dentate gyrus and the posterior pyramidal cell body layer of the CA3 exhibited large undulations in fyn$^{-/-}$ mice (for example see Figure 13, n = 9) that was not present in wild type hybrid animals (for example see Figure 12, n = 7).

3.5. Input-output relationship in fyn$^{-/-}$ mice

To establish if the input to the CA1 region was disturbed the relationship between stimulus intensity (that is, the effect of recruiting more Schaffer collateral fibres) and the initial fEPSP slope was examined. Fibre volley amplitude was plotted against initial fEPSP slope for fyn$^{-/-}$ and wild type hybrid animals. No difference was seen in the spread of points in between the two groups (Figure 14A). The average fEPSP slope showed no significant difference (Table 4). Figure 14B displays the averaged slope of the initial fEPSP plotted against stimulus intensity. No statistical difference was seen in the maximum fEPSP size between the fyn$^{-/-}$ mice (n = 7) and wild type mice (n = 9, P = 0.334, students t-test, see Table 4). A trend for a slightly larger field EPSP slope is apparent in fyn$^{-/-}$ mice that indicated the I/O relationship may be slightly different compared to wild type mice.

Figure 14 **Input-output relationships in fyn knockout and wild type animals (See 3.5 and 3.7.).**

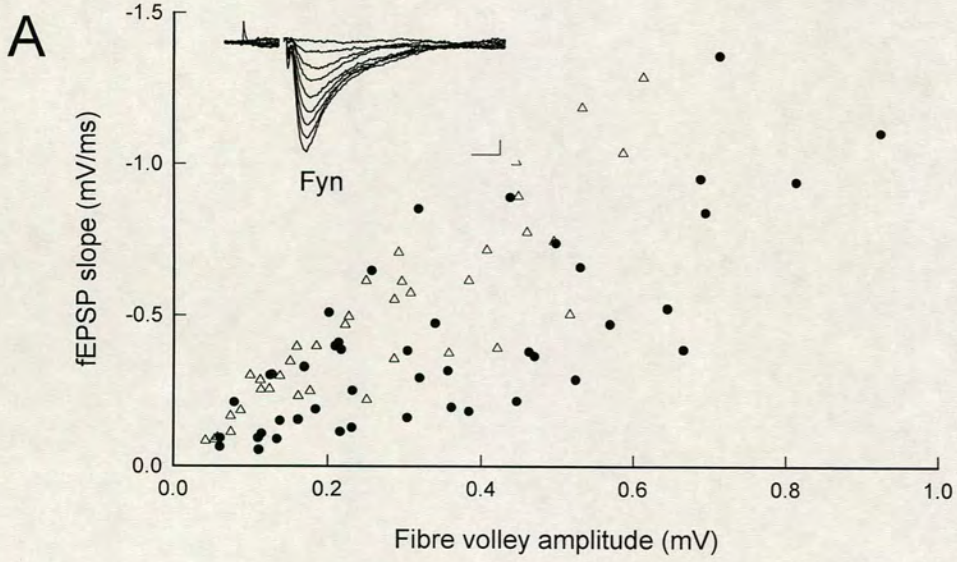
A The fibre volley amplitude plotted against the fEPSP initial slope in animals from the hybrid (C57BL6 x 129Sv) genetic background. No apparent difference in the spread of points is seen. Inset, example traces showing the increasing fEPSP size with increasing stimulus intensity in fyn knockout mice, scale bar, 0.25mV, 10ms.

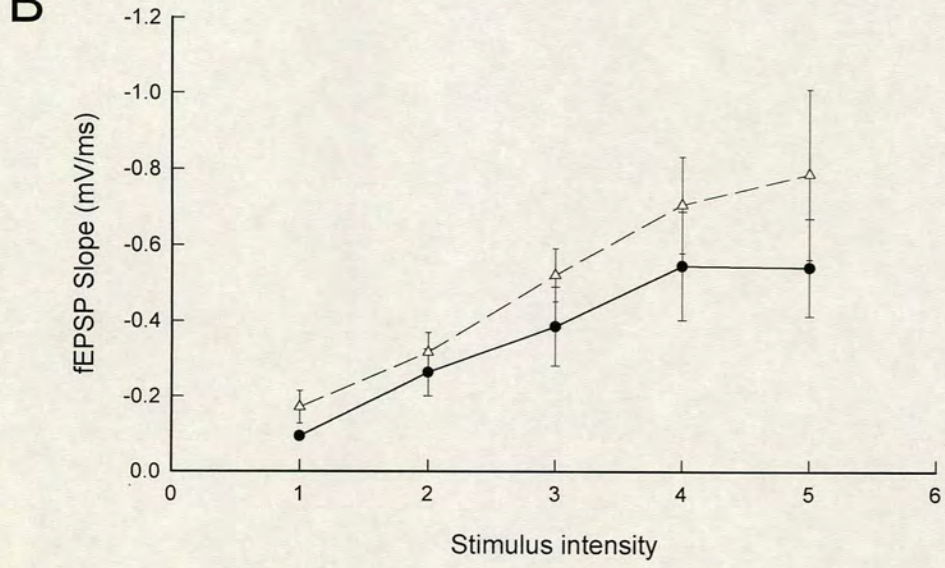
B Averaged results from experiments presented in A. Stimulus intensity (arbitrary units) is plotted against mean fEPSP slope.

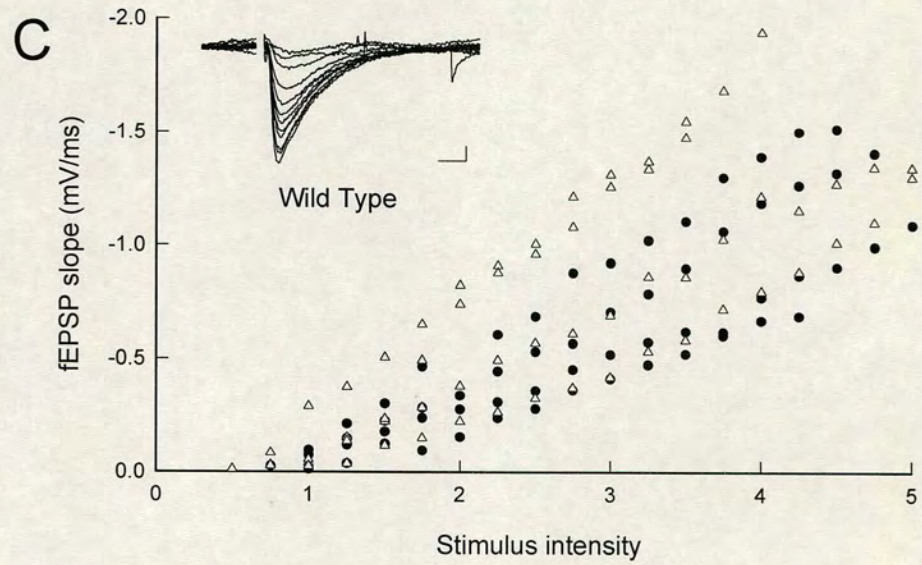
C The stimulus intensity plotted against fEPSP size for inbred 129/Sv animals. No difference was seen between $fyn^{129/Sv-/-}$ and wild type 129/Sv animals. Inset, example traces of increasing stimulus strength in wild type animal, scale bar 0.25mV, 10ms.

D The averaged data set from experiments presented in C (129/Sv inbred strain).

In all graphs fyn animals are presented as white triangles and wild type animals with black circles. Error bars indicate the mean ± standard error.


A
Fyn
fEPSP slope (mV/ms)
Fibre volley amplitude (mV)
-1.5
-1.0
-0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0


B
fEPSP Slope (mV/ms)
Stimulus intensity
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0
1
2
3
4
5
6


C
Wild Type
fEPSP slope (mV/ms)
Stimulus intensity
-2.0
-1.5
-1.0
-0.5
0.0
0
1
2
3
4
5

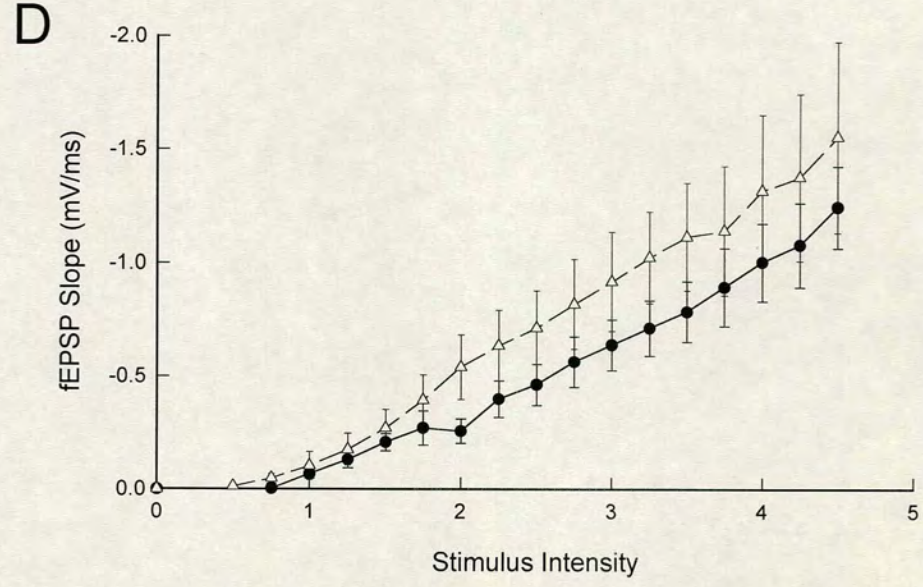

D
fEPSP Slope (mV/ms)
Stimulus Intensity
-2.0
-1.5
-1.0
-0.5
0.0
0
1
2
3
4
5

Table 4 – Input-output relationship for $fyn^{-/-}$ and wild type hybrid animals

Stimulus Intensity	*Wild Type EPSP Slope (mV/ms)*	*Wild Type S.E.M.*	*Fyn EPSP Slope (mV/ms)*	*Fyn S.E.M.*
1	-0.094	0.001	-0.171	0.044
2	-0.263	0.062	-0.317	0.053
3	-0.386	0.105	-0.522	0.070
4	-0.546	0.143	-0.706	0.126
5	-0.542	0.129	-0.787	0.224

Table 5 – Input-output relationship for $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

Stimulus Intensity	*Wild Type EPSP Slope (mV/ms)*	*Wild Type S.E.M.*	*Fyn EPSP Slope (mV/ms)*	*Fyn S.E.M.*
1	-0.066	0.018	-0.103	0.062
2	-0.258	0.053	-0.542	0.143
3	-0.641	0.112	-0.920	0.217
4	-1.006	0.170	-1.319	0.333
5	-1.091	0.001	-1.799	0.479

Figure 12 **Example photographs of the hippocampal morphology in wild type animals on the hybrid genetic background (C57BL6 x 129/Sv).**

Upper panel Anterior hippocampus

Lower panel Posterior hippocampus.

Note that the disturbed architecture apparent in the fyn knockouts is not present.

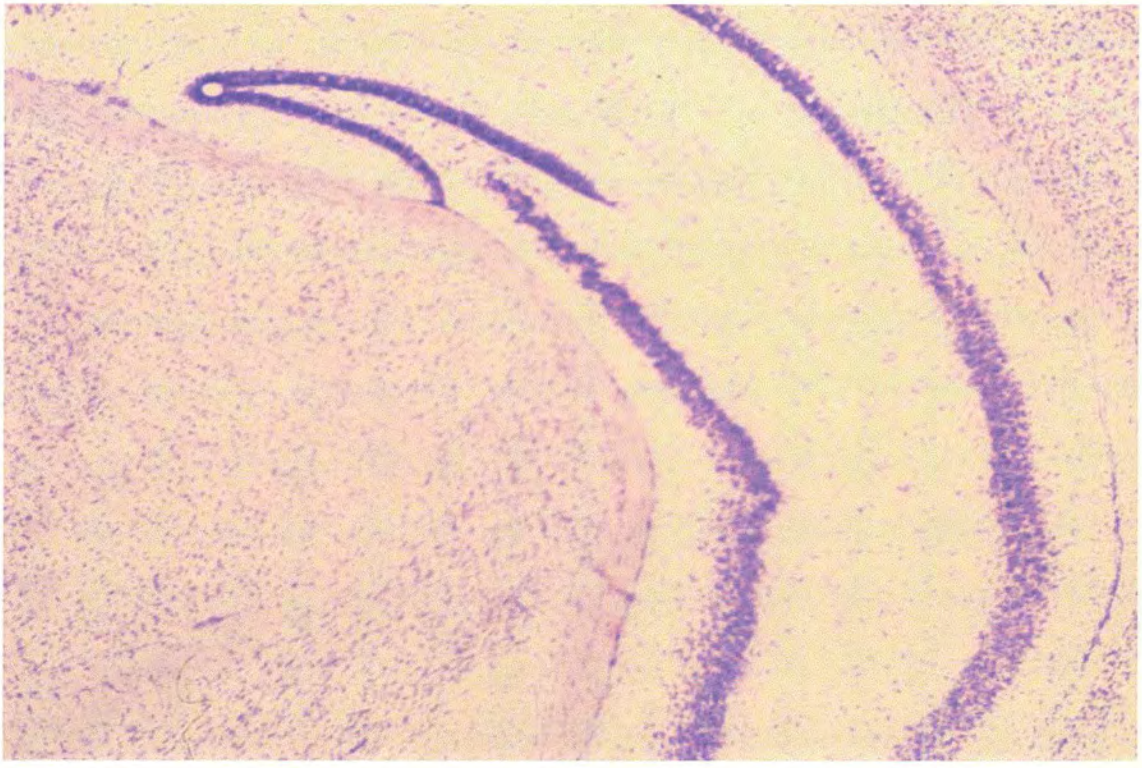
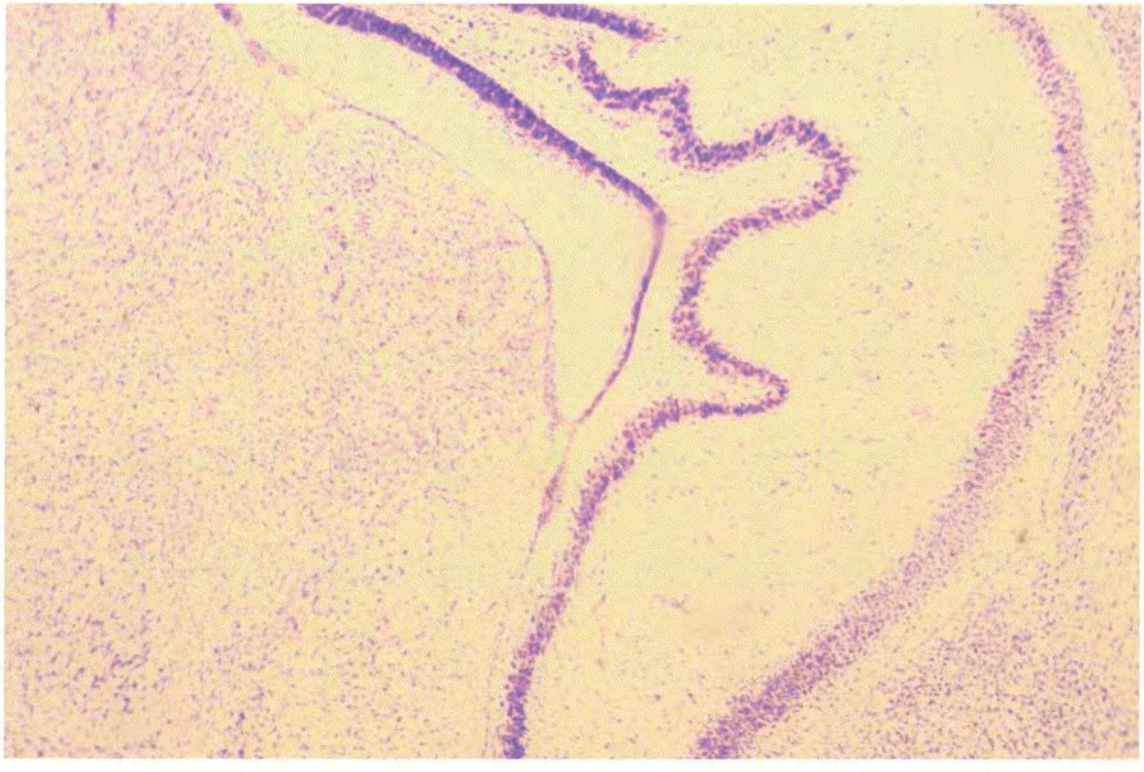


Figure 13 **Example photographs of the disturbed hippocampal morphology in $fyn^{-/-}$ animals on the hybrid genetic background (C57BL6 x 129/Sv).**

Upper panel Anterior hippocampus.

Lower panel Posterior hippocampus.

Note the undulations in the dentate gyrus and CA3 cell body layers.



3.6. $Fyn^{129/Sv-/-}$ long-term potentiation experiments

A second series of LTP experiments were performed on mice from a pure 129/Sv inbred strain ($fyn^{129/Sv-/-}$ mice) as the genetic background of the animals was a potential difference in experimental protocol between the studies reported above and the Grant et al. study. Four $fyn^{-/-}$ mice (3 males, 1 female) were obtained from The Jackson Laboratory (U.S.A.) and backcrossed to generate a new colony of 129/Sv animals. This procedure took around 9 months to generate animals necessary for the LTP experiments. The animals had to be kept in quarantine initially and breeding between homozygote offspring proved to be difficult. An additional length of time was needed to allow the animals to mature to the required age (over 14 weeks, see discussion and Kojima et al., 1997).

Initial experiments were performed using the 25% stimulus level, as the impairment in LTP should be most readily visible at this stimulation intensity. Pooled data sets and an example of an individual experiment for both wild type 129/Sv and $fyn^{129/Sv-/-}$ mice is shown in 15. A significant difference was seen in $fyn^{129/Sv\ -/-}$ mice (108 ± 6 %, n = 10) as compared to wild type 129/Sv mice (159 ± 12 %, n = 15) at 60 minutes post tetanus (P = 0.001). This difference was also apparent during the STP phase, with a significant difference between $fyn^{129/Sv-/-}$ mice (135 ± 6 %) and wild type 129/Sv mice (188 ± 17 %, P = 0.01). However no significant difference was seen in the levels of PTP exhibited between the two groups of mice (235 ± 29 % $fyn^{129/Sv-/-}$, 329 ± 41 % wild type 129/Sv, P = 0.1).

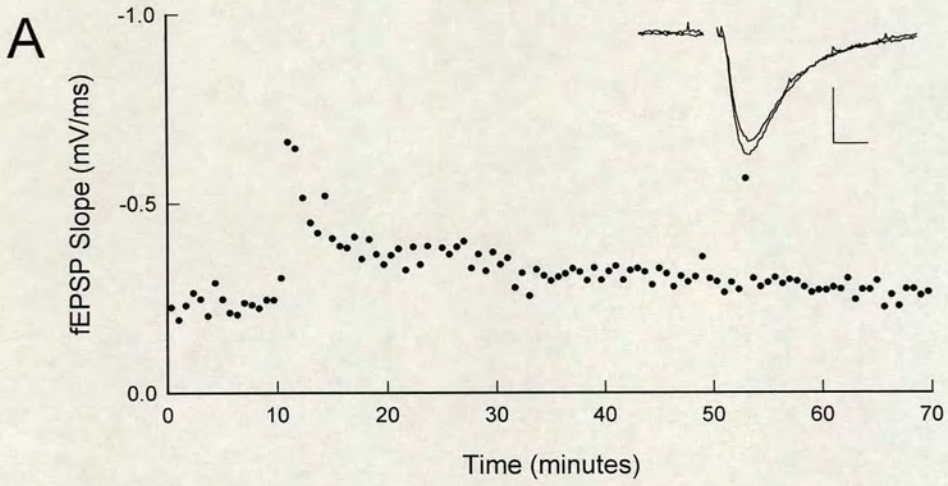
Experiments were repeated at the 75% level of stimulation intensity. Example traces and graphs from individual experiments and the pooled data sets shown in Figure 16. Once again a significant difference was seen between the two groups of mice at sixty minutes post-tetanus (116 ± 6 %, n = 9, $fyn^{129/Sv-/-}$, 154 ± 14 %, n = 12, wild type 129/Sv, P = 0.02). However, STP showed no significant difference (160 ± 11 % $fyn^{129/Sv-/-}$, 187 ± 13 % wild type 129/Sv, P = 0.1) between the genotypes.

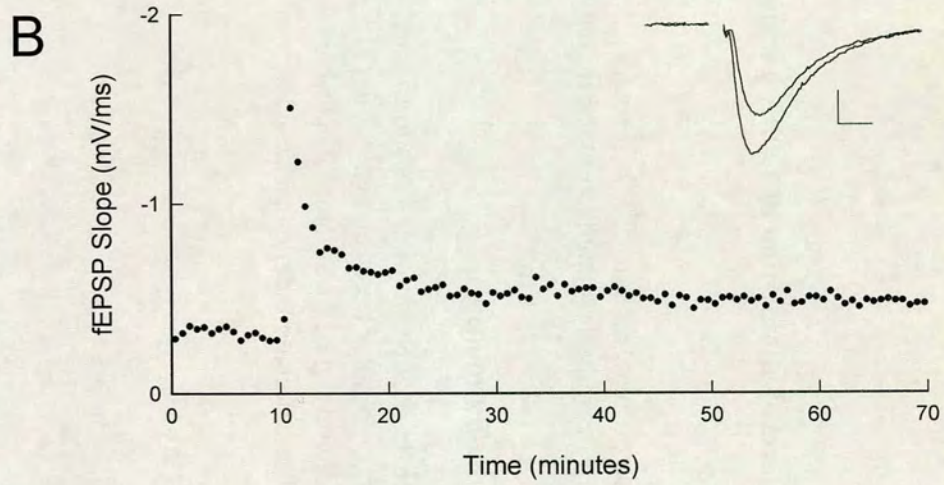
Figure 15 **LTP experiments in fyn$^{129/Sv-/-}$ and wild type 129/Sv animals at the 25% level of stimulation (See 3.6.)**

A Individual example of experiment for fyn$^{129/Sv-/-}$ are shown. Insets, example traces of fEPSPs both pre and post tetanus. Scale bars are 0.25mV, 5ms in both cases.

B As above for wild type 129/Sv animals

C The averaged pooled data from fyn$^{129/Sv-/-}$ (n=10, white triangles) and wild type 129/Sv (n=15, black circles). A significant difference was seen between fyn$^{129/Sv-/-}$ (108 ± 6 %) compared to wild type animals (159 ± 12 %) at sixty minutes post tetanisation. Error bars indicate the mean ± standard error.


A
fEPSP Slope (mV/ms)
-1.0
-0.5
0.0
0
10
20
30
40
50
60
70
Time (minutes)


B
fEPSP Slope (mV/ms)
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)

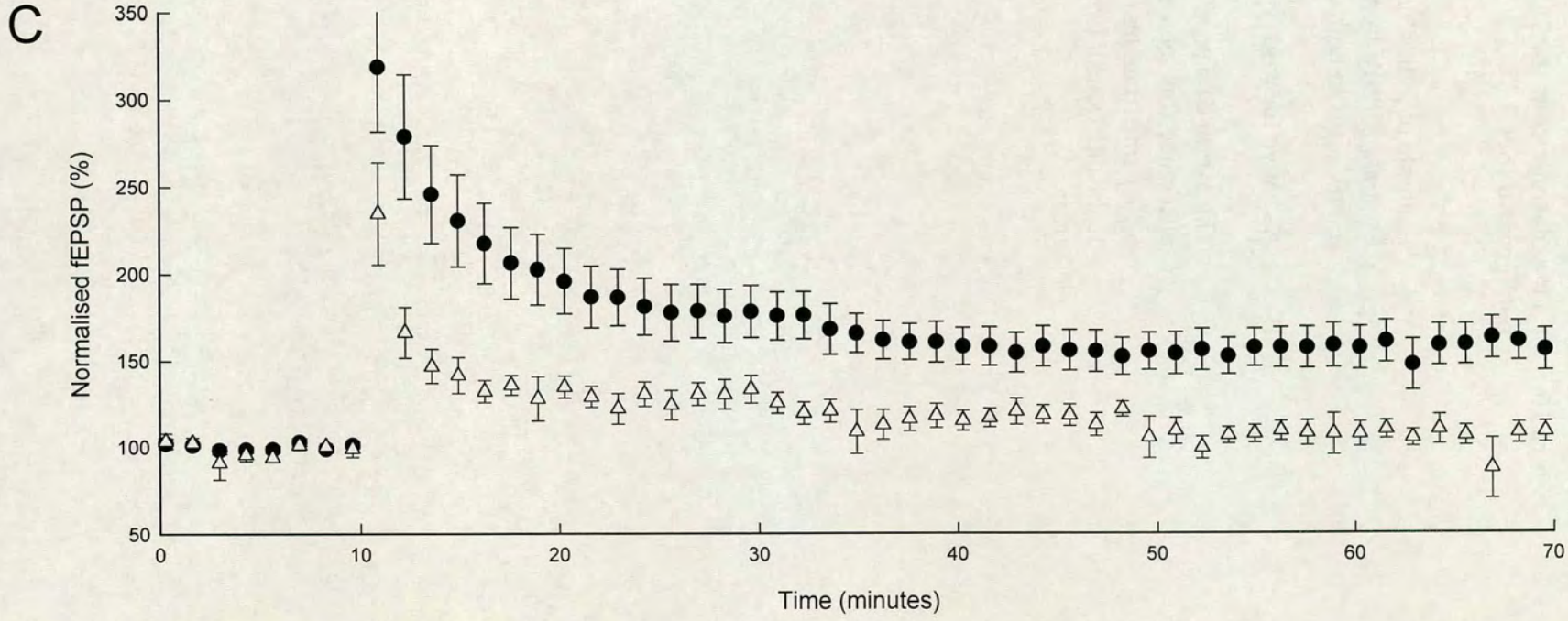
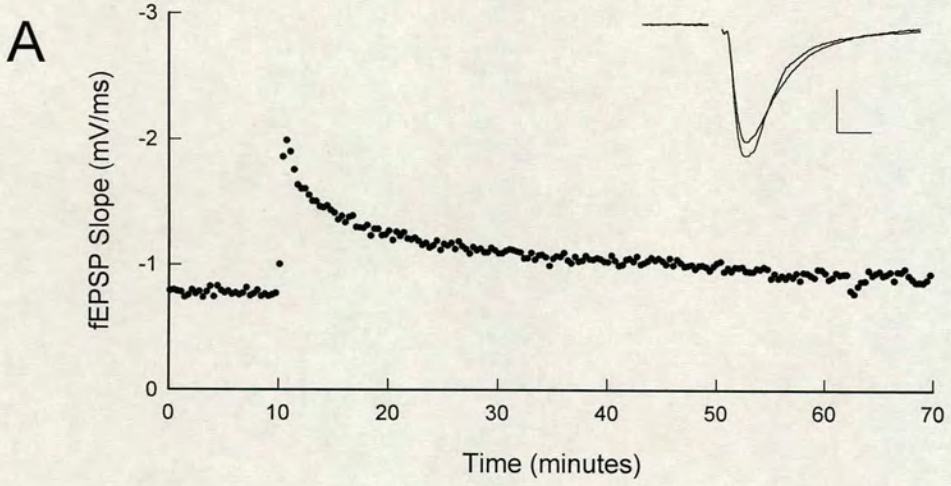

C
Normalised fEPSP (%)
350
300
250
200
150
100
50
0
10
20
30
40
50
60
70
Time (minutes)

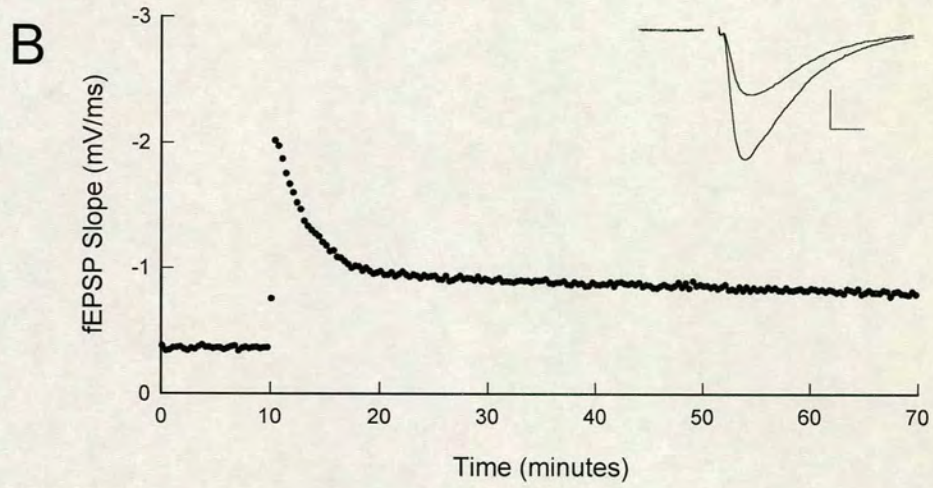
Figure 16 **LTP experiments at the 75% level of stimulation in 129/Sv genetic background (See 3.6).**

A Example of individual experiment for $fyn^{129/Sv-/-}$. Insets, example fEPSPs from before and after tetanisation, Scale bars 0.5mV, 5ms in both cases.

B As above for wild type 129/Sv animals

C The averaged data set for the 129/Sv animals. A significant difference was seen at sixty minutes between $fyn^{129/Sv-/-}$ (116 ± 6 %, white triangles) and wild type 129/Sv animals (154 ± 14 %, black circles). Error bars indicate the mean ± standard error.


A
fEPSP Slope (mV/ms)
-3
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)


B
fEPSP Slope (mV/ms)
-3
-2
-1
0
0
10
20
30
40
50
60
70
Time (minutes)

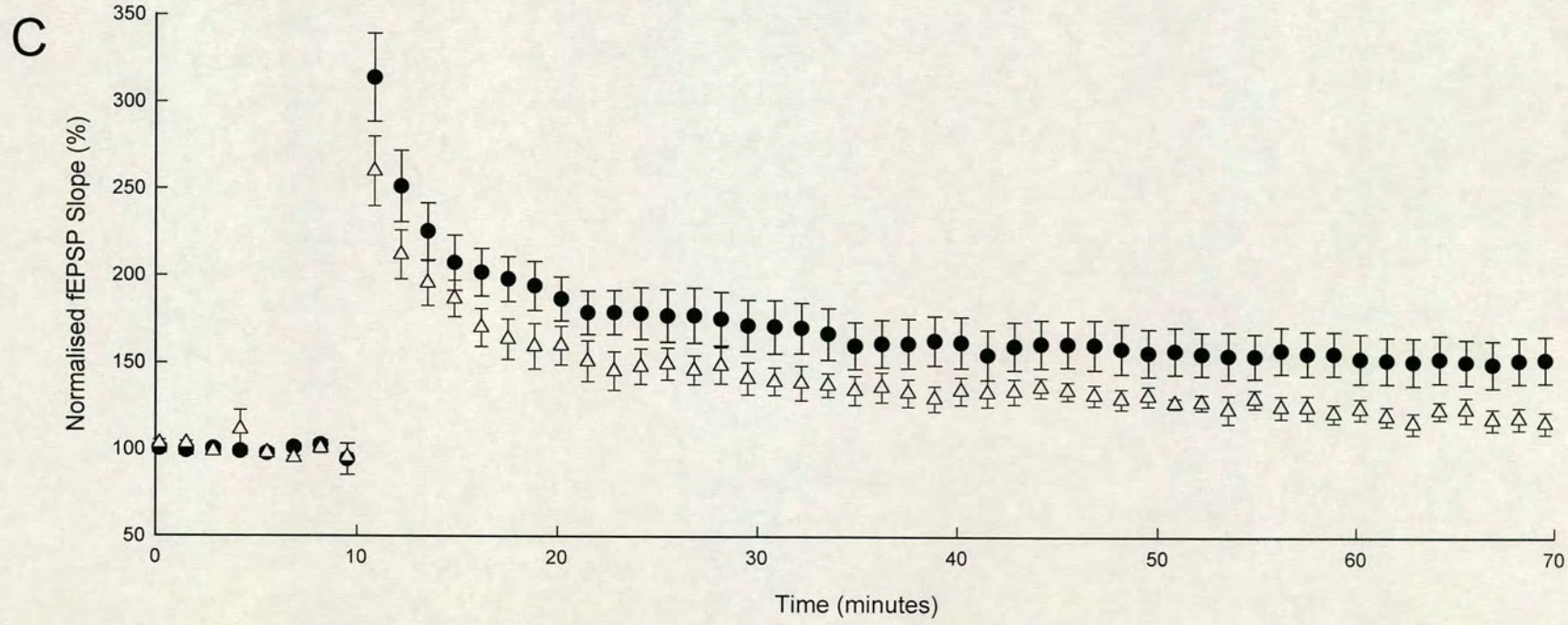

C
Normalised fEPSP Slope (%)
350
300
250
200
150
100
50
0
10
20
30
40
50
60
70
Time (minutes)

Table 6 – Summary of long-term potentiation results in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

Experiment	*LTP ± S.E.M.*	*N*	*Significant*	*P value*
129Sv Wild Type 25% Submerged	158.7 ± 12.3	15	Yes	0.0014
129Sv Fyn 25% Submerged	107.9 ± 5.8	10		
129Sv Wild Type 75% Submerged	154.2 ± 13.6	12	Yes	0.0224
129Sv Fyn 75% Submerged	116.1 ± 5.9	9		
Grant et al. Study 1mV Wild Type	177.5 ± 2.9	7	Yes	<0.0001
Grant et al. Study 1mV Fyn	90.5 ± 4.5	11		
Grant et al. Study 25% Wild Type	149.6 ± 18.2	9	No	0.0611
Grant et al. Study 25% Fyn	108 ± 7.6	8		
Grant et al. Study 75% Wild Type	168.5 ± 11.6	12	Yes	0.0415
Grant et al. Study 75% Fyn	133 ± 9.3	8		

Notably, however, potentiation after the tetanus (PTP time point) was significantly different in the two groups of mice (254 ± 16 % $fyn^{129/Sv-/-}$ mice, 339 ± 35 % wild type 129/Sv mice, P = 0.04). These results are summarised in Table 6, along with the data from the Grant et al., 1992 study.

3.7. Input-output relationship in 129/Sv genetic background

The relationship between stimulus intensity and the initial fEPSP slope was examined in the 129/Sv inbred mice. Figure 14C displays the slope of the initial fEPSP plotted against stimulus intensity for all animals. The fEPSP slope values were averaged and plotted against stimulus intensity (Figure 14D). No difference was noted at between the two groups, and the maximum fEPSP size generated in wild type 120/Sv mice was not statistically different from that seen in $fyn^{129/Sv-/-}$ mice (P = 0.0598. students t-test, see Table 5).

3.8. Hippocampal morphology in $fyn^{129/Sv-/-}$ mice

Gross hippocampal morphology was also analysed in the 129/Sv background. A similar pattern of disruption to the cell body layers of the anterior dentate gyrus and the posterior CA3 was seen in the $fyn^{129/Sv-/-}$ mice (n = 12). This abnormal arrangement of cells was not seen in any wild type 129/Sv mice (n = 8). Examples of both $fyn^{129/Sv-/-}$ and wild type 129/Sv mice sections are shown in Figures 17 and 18.

Figure 17 **Example photographs of the hippocampal morphology in wild type animals on the inbred 129/Sv genetic background.**

Upper panel Anterior hippocampus

Lower panel Posterior hippocampus.

Note that the disturbed architecture apparent in the fyn knockouts is not present. (See 3.8.)

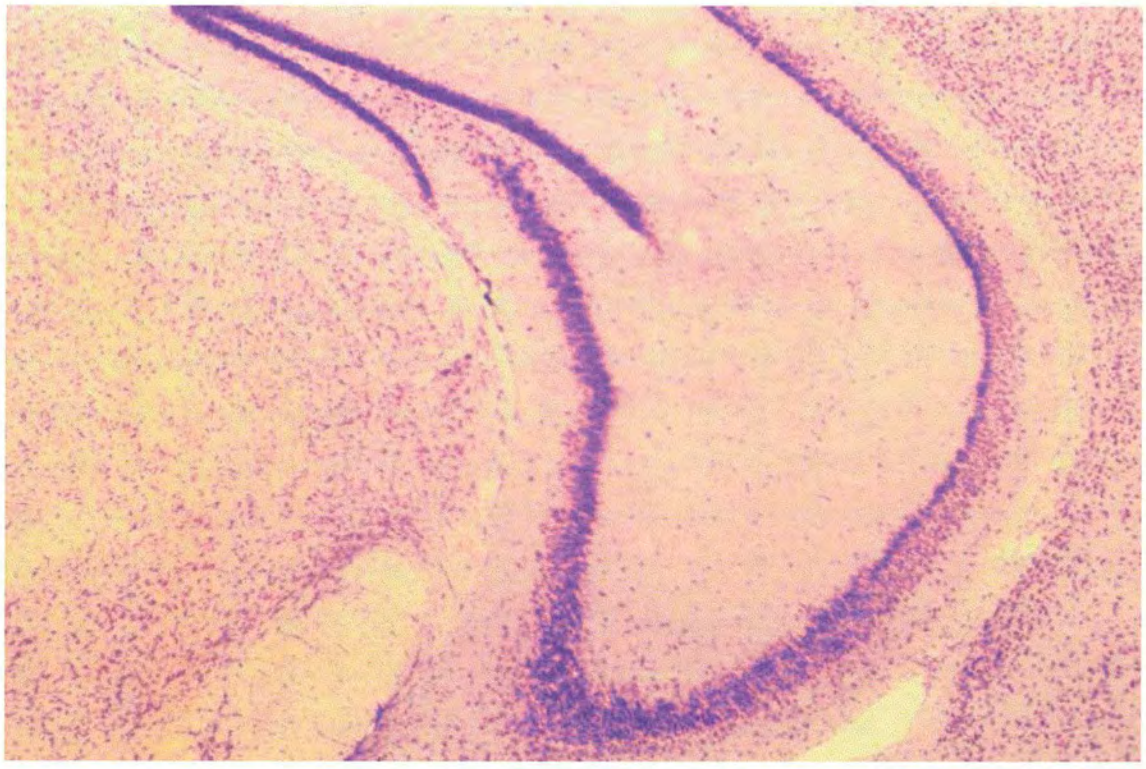
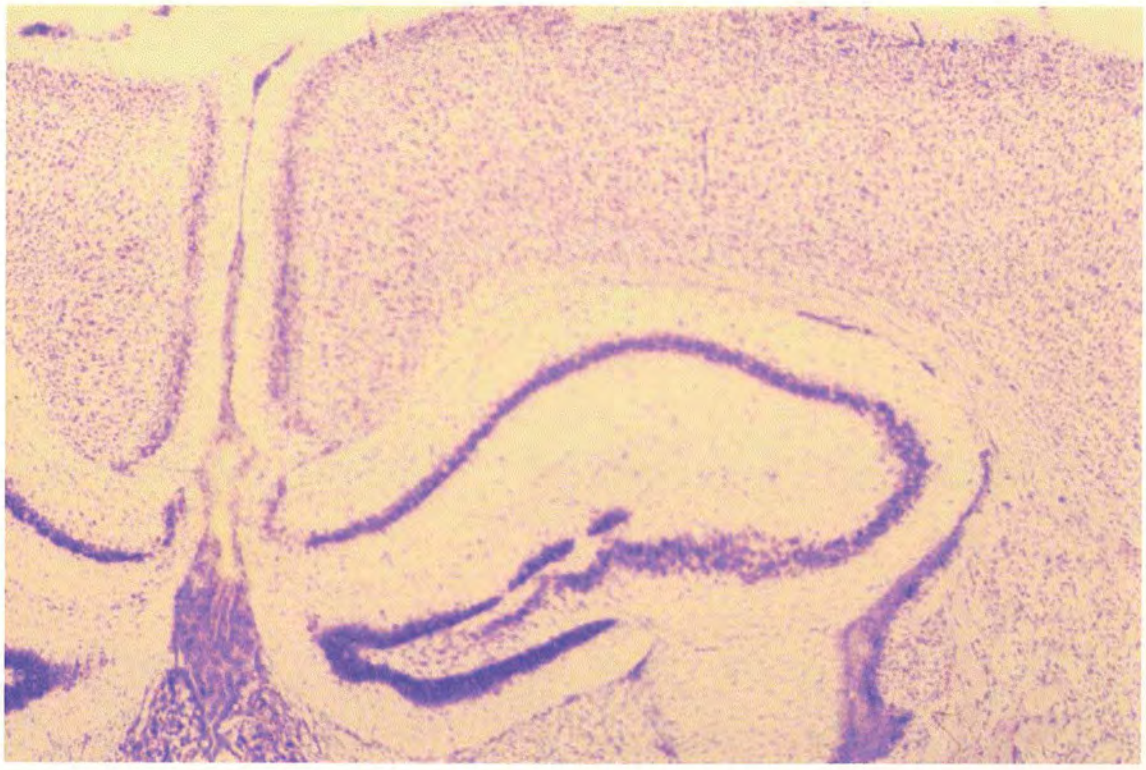


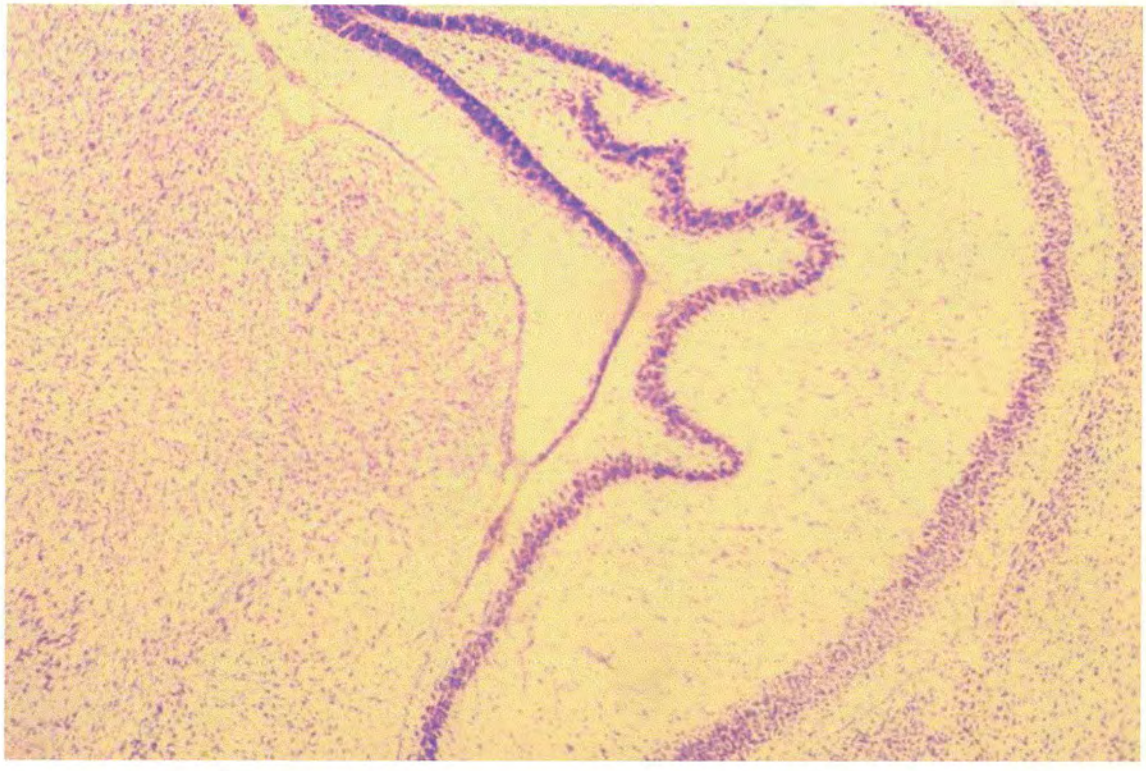
Figure 18 **Example photographs of the disturbed hippocampal morphology in $fyn^{129/Sv-/-}$ animals on the 129/Sv inbred genetic background.**

Upper panel Anterior hippocampus

Lower panel Posterior hippocampus.

Note the undulations in the dentate gyrus and CA3 cell body layers (See 3.8.).





Chapter IV

Discussion

Long-term potentiation, morphology and basal synaptic transmission

4.1. Introduction

The induction and maintenance of NMDA receptor mediated LTP in the hippocampus requires the activation and regulation of multiple ion channels and many intracellular signalling pathways, triggered by the rise in intracellular calcium through the NMDA receptor. The biochemical signals that mediate this process have recently come under the scrutiny of molecular biology, which has implicated a number of molecules important for this form of synaptic plasticity. These include protein kinases (tyrosine and serine / threonine), phosphatases, scaffolding proteins, structural proteins and regulators and the subunits of ion channels themselves.

Evidence through pharmacological intervention (O'Dell et al., 1991, Wang and Salter, 1994) and through the use of genetic knockout and transgenic mice (Grant et al., 1992, Kojima et al., 1997, Lu et al., 1999, Narisawa-Saito et al., 1999) has implicated the src family of tyrosine kinases in the induction of LTP in the hippocampus. In this study, knockout mice were used to investigate synaptic plasticity in mice lacking molecules postulated to be important in signalling cascades downstream of the NMDA receptor.

4.2. $p140^{ras\text{-}GRF}$ knockout mice long-term potentiation experiments

In 1990 Wolfman and Macara discovered a factor which accelerated the rate of GDP release from $p21^{ras\text{-}GDP}$ which they termed $p140^{ras\text{-}GRF}$ (*ras*-guanine-nucleotide-releasing-factor). $p140^{ras\text{-}GRF}$ contains an IQ motif which allows it to bind calcium / calmodulin and other related calcium binding proteins. Calcium influx has been shown to enhance the activity of $p140^{ras\text{-}GRF}$ and subsequently $p21^{ras}$ (Farnsworth et al., 1995). The mechanism by which the calcium / calmodulin complex activates $p140^{ras\text{-}GRF}$ remains to be determined. It has been proposed that calcium mediated $p21^{ras}$ activation may play a role in calcium dependent forms of plasticity, such as LTP and LTD (Kennedy, 1989). A recent study demonstrates a novel Ras-GTPase (p135 SynGAP) which is regulated by CaMKII, and localised to the NMDA receptor

signalling complex by PSD-95, further indicating a role of the $p21^{ras}$ signalling cascade in long term potentiation (Chen et al., 1998). To study the role of $p140^{ras\text{-}GRF}$ in brain function and synaptic plasticity, a $p140^{ras\text{-}GRF}$ knockout mouse was engineered (Brambilla et al., 1997).

Tetanically induced hippocampal LTP in $p140^{ras\text{-}GRF}$ knockout mice was indistinguishable to wild type mice in area CA1 of the hippocampus. This implies that $p140^{ras\text{-}GRF}$ is not required for this form of plasticity in this brain region. $p21^{ras}$ is involved in the signalling of growth factor receptors and non-receptor tyrosine kinases to the MAPK kinase cascade that can lead to gene expression (Ginty et al., 1994). The $p21^{ras}$ regulatory protein $p140^{ras\text{-}GRF}$ (Ras-neuronal-specific-guanine-nucleotide-exchange factor (Ras-GRF / CDC25Mm) is exclusively expressed postsynaptically and its activity is up-regulated when bound to calcium / calmodulin (Farnsworth et al., 1995, Wei et al., 1993, Ferrari et al., 1994). This factor therefore provides a mechanism by which calcium influx can activate the $p21^{ras}$ - MAPK pathway (Finkbeiner and Greenberg, 1996). Calcium dependent mechanisms that activate the processes of transcription and translation are of interest in long term potentiation mechanisms as the increase in synaptic efficacy associated with late LTP requires protein synthesis (Frey et al., 1988).

Ras genes are ubiquitous, and have been found in all eukaryotic cells. They code for a 21kDa membrane associated protein - $p21^{ras}$. These proteins have been shown to play a crucial role in receptor activated signal transduction pathways. $p21^{ras}$ specifically binds guanine nucleotides, the active complex being $p21^{ras\text{-}GTP}$. The rate of hydrolysis of $p21^{ras\text{-}GTP}$ to $p21^{ras\text{-}GDP}$ is rapid and is accelerated by the action of *ras*-GAP (*ras* GTP-ase activating protein). At physiological concentrations of Mg^{2+}, the rate of GDP release from $p21^{ras\text{-}GDP}$ is minimal, thus $p21^{ras}$ tends to remain in the inactive state ($p21^{ras\text{-}GDP}$). Growth factor receptors and non-receptor tyrosine kinases can both activate $p21^{ras}$ through the Grb2-SOS complex, which accelerates the release of GDP from $p21^{ras\text{-}GDP}$ allowing GTP to bind. The active $p21^{ras\text{-}GTP}$ complex initiates a phosphorylation cascade of: MAPKKK (Raf) / MAPKK / MAPK which modulates the levels of translation and transcription in the cell (see Figure 2, Chapter

1). An important consideration in this series of experiments is that LTP was observed for only one hour after induction. If $p140^{ras\text{-}GRF}$ is involved in calcium dependent regulation of translation and transcription, a deficiency in the late phase of LTP may occur. Changes in transcription are known to occur 3 - 4 hours after the tetanic induction of LTP (Frey et al., 1988), therefore late phase LTP experiments (6 - 8 hours) may uncover a phenotype in these animals. Additionally, no difference between wild type and $p140^{ras\text{-}GRF}$ knockout mice was observed in the Morris water maze and in a radial arm maze, both of which depend on normal hippocampal function (Brambilla et al., 1997).

In contrast, *in vitro* amygdaloid LTP was reduced in $p140^{ras\text{-}GRF}$ mutants and behaviors dependent on the amygdala were significantly impaired (fear conditioning tests, Brambilla et al., 1997). These data suggest that although there is considerable evidence for the role of the Ras / Raf / MAPK pathway in hippocampal LTP (reviewed in Orban et al., 1999, English and Sweatt, 1996), the loss of $p140^{ras\text{-}GRF}$ can either be compensated for or is redundant in the early phase (up to one hour) of tetanically induced NMDA receptor dependent LTP in area CA1 of the hippocampus. As such, this molecule appears to be more important for behaviors involving fear conditioning and in the induction of LTP in the amygdala. There are known differences in LTP seen in the hippocampus and the amygdala. For example, certain pathways in the amygdala show non-NMDA receptor dependent LTP (Chapman and Bellavance, 1992) and (as muscarinic AChRs are highly expressed in the basolateral amygdala) muscarinic antagonists can block LTP in this structure (Watanabe et al. 1995). Interestingly, muscarinic receptors can activate $p140^{ras\text{-}GRF}$ and produce an increase in its phosphorylation level, so this pathway may be more physiologically relevant to amygdala function (Mattingly and Macara, 1996).

4.3. $Fyn^{-/-}$ / $FAK^{+/-}$ long-term potentiation experiments

Focal adhesion kinase (FAK, $p125^{fak}$) is responsible for the maintenance of extracellular matrix to cell contacts in non-neural cells, but has an unknown function

in neurones although it is expressed at high levels in the adult CNS. FAK is a potential substrate for fyn tyrosine kinase and binds both fyn and src which mutually regulates activity. In fyn knockout mice, FAK is seen to be hypo-phosphorylated, thus a mouse lacking one FAK allele and lacking a FAK regulatory molecule (i.e. fyn) were created.

Experiments were performed to determine if mice with a significantly reduced level of functional focal adhesion kinase (FAK) had impaired NMDA receptor dependent LTP. No significant difference was seen between wild type mice and $fyn^{-/-}$ / $FAK^{+/-}$ mice (Figure 8). Mice lacking fyn tyrosine kinase have been reported to show a reduced threshold for LTP induction (Grant et al., 1992). In the $fyn^{-/-}$ / $FAK^{+/-}$ mice this impaired LTP was apparently reversed by the deletion of one FAK allele. This could be interpreted as an indication that a loss of a significant amount of functional FAK produces an enhancement of LTP. However, these animals share the same hybrid genetic background as the $fyn^{-/-}$ mice (C57BL6 x 129/Sv, detailed below) and genetic background issues encountered with these $fyn^{-/-}$ animals precludes any firm conclusions being drawn as to the role of FAK in LTP from this study (see 4.4.6). Additionally animals used in this study express a small functional amount of FAK, and this could also mask any potential knockout phenotype.

A novel brain specific isoform of FAK was identified during the course of these experiments, termed FAK (+) (Burgaya et al., 1996, Derkinderen et al., 1996). A brain specific knockout mouse FAK $(+)^{-/-}$ is currently under development in our laboratory and a decision was made not to continue the LTP work on the $fyn^{-/-}$ / $FAK^{+/-}$ mice until a more suitable animal for investigating the role of FAK in the induction of LTP was made available. During the preparation of this manuscript, FAK(+) deficient embryonic stem (ES) cells have been successfully introduced to produce viable FAK(+) deficient chimeras, and the F_1 offspring, displaying a FAK $(+)^{-/-}$ genotype appear normal and healthy. These mice can be used to address the role of FAK in LTP in area CA1 of the hippocampus. This said, a report of mice with a $fyn^{-/-}$ / $FAK^{+/-}$ genotype has been published by another group which reported abnormal skin growth in old animals (> 8 months of age). These animals were

generated by replacing the fyn allele with a *lacZ* gene, and mating with FAK heterozygotes (Llic et al, 1997). However, no apparent differences were seen in skin condition in animals from the Edinburgh colony of $fyn^{-/-}$ / $FAK^{+/-}$ mice, although few animals were maintained over 8 months of age.

The apparent lack of effect of this additional mutation, led the author to re-establish the reported impairment of LTP in $fyn^{-/-}$ mice.

4.4. $Fyn^{-/-}$ long-term potentiation experiments

Fyn ($p59^{fyn}$) tyrosine kinase has been implicated as an important molecule in the process of LTP in the hippocampus through analysis of a knockout animal (Grant et al., 1992). In re-analysing these animals several caveats of these initial experiments became apparent, which have been addressed in this thesis. The issues raised in this study are discussed below.

4.4.1. Replication of Grant et al. 1992 long-term potentiation experiments

4.4.1.a. Maximal fEPSP slope determination

In the Grant et al. study, the maximal fEPSP slope / size was determined by increasing the stimulation until the response saturated, displaying a large fEPSP population spike complex (Figure 5C, Chapter 2). This method of determining the maximal fEPSP slope is not considered suitable by the author for the type of recordings made in this study. The initial slope of the fEPSP trace generated by extracellular recording in area CA1 is considered to be predominately mediated by a fast AMPA receptor current, and is the usual measurement recorded in an LTP protocol. The development of a large population spike complex to the fEPSP can distort the initial slope measurement that then becomes a combination of the fast AMPA receptor mediated component and an action potential mediated component.

These two components have very different electrophysiological properties and should not be considered in the same measurement. This does mean however that the stimulation used in the data presented in this study is significantly less than that used in the Grant et al. study. A detailed description of how the maximal fEPSP size was determined in this thesis is presented in 2.8.1., Figure 5A). The author is aware the measurement of the amplitude of the fEPSP population spike is a recognised method of determining synaptic efficacy, but believes that the combination of both initial fEPSP slope measurements with a combined fEPSP population spike is unsuitable for this type of experiment.

4.4.1.b. Recording chamber type

An interface style of recording chamber was used to generate LTP in the Grant et al. study, whilst a submerged system was used for initial experiments in the $fyn^{-/-}$ mice. Whilst it is not immediately obvious why this should make a substantial difference, there is evidence that a small reduction (0.15 ± 0.11 pH units) occurs in the pH level of the extracellular medium in hippocampal slices when moved from an interface type of recording configuration to a submerged type (Bortolotto et al., 1995). In this respect pH changes can affect both NMDA receptor activity and glutaminergic and GABA-ergic synaptic transmission which will affect the probability of inducing LTP.

To determine if the style of recording chamber had any effect on the level of potentiation seen following tetanic stimulation experiments were repeated in an interface style chamber. Results from experiments at the lowest level of stimulation (25% maximal fEPSP slope) show that the type of recording chamber had no significant effect on the levels of potentiation seen. This was therefore discounted as a possible reason for the loss of phenotype seen in animals from the hybrid background ($fyn^{-/-}$).

4.4.1.c. ACSF composition

The Grant et al study used a modified ACSF, the composition of which is shown in Table 1. The most significant difference in the composition of the 'Grant et al' ACSF compared to the ACSF used throughout this study is the increased potassium ion concentration. This would tend to depolarise the cells in the slice to a slightly lower resting membrane potential and could possibly influence the level of activation of CA1 pyramidal cell NMDA receptor currents and the generation of action potentials in the CA1 inputs. However, no difference in the level of potentiation was seen when this ACSF composition was used in conjunction with the interface style of recording chamber. All subsequent experiments performed after this study use the 'Standard' ACSF composition detailed in Table 1.

4.4.2. Levels of potentiation and stimulation in extracellular LTP experiments

Another issue of note is that the recording technique applied to this study, that is extracellular fEPSP recordings of LTP, may not be a sensitive enough assay to discern an effect of a genetic ablation of a modulatory molecule. The variation associated with averaged level of potentiation of extracellular fEPSP slope is generally in the region of 5-20% (S.E.M.). Therefore to statistically demonstrate a significant effect, the parameter under study must show a relative change greater than the standard deviation of the data set. To date several studies have shown that genetic ablation of a particular molecule leads to the complete lack of LTP which can be easily discerned using this style of experiment (e.g. CaMKII, Silva et al., 1992). In $fyn^{-/-}$ / $FAK^{+/-}$ mice and $fyn^{-/-}$ mice however, where the molecule in question is not an absolute requirement for the process of LTP induction but rather a modulator of the level of induction threshold, small effects may be overshadowed by the significant averaging inherent in this type of recording or the strong induction protocol used (2x100Hz). In re-analysis of the figures stated in the Grant et al. study by the author of this thesis, a significant difference does exist between $fyn^{-/-}$ mice and wild type mice at the 1mV (where the stimulation strength was set to obtain a fEPSP

with an amplitude of 1mV) and 75% levels of stimulation ($P = < 0.0001$ and $P = 0.04$ respectively). However, the result generated at the 25% level of stimulation was not statistically different from the wild type control group ($P = 0.06$) indicating that the reported reduction in LTP at this stimulus intensity could have been accounted for by variance in the sample population. This effect is not noted in the Grant et al. paper.

The purpose of using the two different stimulus intensities was to indicate a change in the level of threshold for LTP induction in $fyn^{-/-}$ mice. However this strategy is at odds with the very strong induction protocol used (2 x 100Hz trains), and may explain why a clear result was not obtained in the Grant et al. study. The author of this thesis is unaware of any other study in which 2 trains of 100Hz stimuli is used to induce LTP and can offer no explanation why the authors of this paper did not use the established single train to induce LTP.

Interestingly in the Grant et al. study there was no significant difference between the results at the 25 or 75 % level of stimulation ($P = 0.4$ wild type; $P = 0.06$ $fyn^{-/-}$, again re-analysed by the author of this thesis), indicating that the level of stimulation had no effect on the final level of potentiation. Wild type mice in fact show no significant difference in the final level of potentiation at all three of the stimulus intensities used (25%, 75% and 1mV). $Fyn^{-/-}$ mice only show a significant difference at the 75% - 1mV level ($P = 0.0003$) although this is due to the fact that at the 1mV level of stimulation, $fyn^{-/-}$ mice actually show an average depression of around 10% sixty minutes after tetanisation. The results generated in this thesis also show no significant effect of stimulation intensity (either 25% or 75%) on the final level of potentiation. This effect was apparent in both genetic backgrounds tested ($P = 0.3$ $fyn^{-/-}$ hybrid mice, $P = 0.7$ wild type hybrid mice, $P = 0.3$ $fyn^{129/Sv-/-}$ mice, $P = 0.8$ wild type 129/Sv mice).

4.4.3. Input–output relationship

The main glutamatergic excitatory synaptic inputs to the CA1 area of the hippocampal slice are the Schaffer collaterals, the axons of the CA3 pyramidal cells, which form en-passant synapses with CA1 basilar and apical dendrites. Changes in the electrophysiological or morphological properties of these cells or inputs could have major effects on any responses recorded from the dendritic arbor of the CA1 pyramidal cells. In $fyn^{-/-}$ mice there is a known defect in the CA3 cell body layer (see below and Grant et al., 1992), that may disrupt the development of normal hippocampal circuitry in these mice. In the Grant et al. study, basal synaptic transmission was determined to be normal on the basis of maximal fEPSP size and the ratio of PPF at 50ms.

In this study the relationship between stimulus strength and fEPSP slope was used as a measure of basal synaptic transmission and the integrity of the CA3 Schaffer collateral / commissural inputs. There was an apparent trend for mice lacking fyn tyrosine kinase from both the hybrid and inbred (129/Sv) lines to show a steeper input-output relationship, however this was not statistically significant.

The I/O relationship will play a major role in determining what conclusions can be drawn from both the PPF and LTP experiments. In the data presented, there is a trend for the fyn mutant mice (from both genetic backgrounds) to show a slightly higher level of fEPSP amplitude as compared to wild type controls. This effect is accounted for in the experimental design by using a relative measure (i.e. 25 %, 50 % and 75 % maximal fEPSP slope) to determine the stimulus intensity level. In each case be it the 25 %, 50 % or 75 % level, both groups of animals are being stimulated at the relative same point on their I/O curve. This in fact is one of the crucial reasons for using a stimulation level which is determined by the individual characteristics of each slice, rather than using a set stimulus strength, which would make it impossible to compare the results slice to slice.

4.4.4. Hippocampal morphology

The reported abnormal arrangement of CA3 and dentate gyrus cells of the $fyn^{-/-}$ mutant mice was apparent at a gross morphological level. In view of the apparent reversal of the LTP phenotype in the $fyn^{-/-}$ hybrid mice the gross morphology of the hippocampal formation was checked, to see if these mice still exhibited the abnormal CA3 and dentate gyrus cell body layers. No difference was noted at a light microscopy level between the abnormal arrangement seen in the Grant et al. study and the mice used in this thesis (both hybrid and 129/Sv strains, Figures 12, 13, 17 and 18). Although animals from the hybrid background do not display the reported LTP phenotype, the morphological abnormality is apparent, indicating that there is still a significant effect of the deletion of fyn in these mice.

4.4.5. Age effects

The age of the mice used in the study was considered in light of a publication detailing the rescue of the fyn impairment in LTP by introduction of fyn cDNA using a CaMKII promoter driven expression system into $fyn^{-/-}$ mice (Kojima et al., 1997). $Fyn^{-/-}$ mice under the age of 14 weeks show normal LTP, and only show the 'blunted' LTP phenotype after 14 weeks of age. All animals used in the present study were over 14 weeks of age and thus this effect cannot explain the apparent loss of the LTP phenotype in the hybrid mice.

The explanation for the reduction of LTP in animals older than 14 weeks of age is due to the compensation of fyn function by another non-receptor tyrosine kinase family member, src. Grant et al., 1995 demonstrate a significant increase in the levels of src kinase activity in the $fyn^{-/-}$ mutants. The level of expression of src in $fyn^{-/-}$ mice is significantly increased in young animals (2-10 weeks), but falls sharply at week 14 (Kojima et al., 1997). This is at the same time period that the loss of LTP is observed in $fyn^{-/-}$ mice. In contradiction to this data is the fact that src knockout mice have been reported to display "normal LTP" in area CA1 of the hippocampus

(Grant et al., 1992) and show no obvious neurological phenotype (Stein et al., 1992). However as no figures are quoted for the level of LTP observed in the src mutants it is difficult to compare, although visual inspection of the data indicates that LTP appears to be slightly elevated compared to wild type mice at the 75% stimulus level (quoted as 169 ± 12 %, see Figure 3D in Grant et al., 1992, a rough estimate for $src^{-/-}$ mice is ≈ 188%). Src is apparently able to compensate for the loss of fyn in $fyn^{-/-}$ mice in young animals (Grant et al., 1995, Kojima et al. 1997). These data suggest a role for src in the induction of LTP in area CA1 of the hippocampus in $fyn^{-/-}$ mice, and may indicate the requirement for both kinases in wild type animals. It is important to note the results published in the Kojima et al. study were also generated from mice with a hybrid background. The CaMKII driven transgene expressing fyn was introduced to the pronuclei of fertilized eggs from C57BL/6J / CBA / F_1 mice. Fyn rescue mouse lines were generated from crossing heterozygous fyn knockout mice with a C57BL/6 x 129/Sv background with transgenic F_1 individuals.

The evidence presented by Kojima et al. did confirm that the LTP impairment seen in $fyn^{-/-}$ mice was specifically due to the lack of fyn kinase and was not accounted for by the disturbed hippocampal architecture. The developmental abnormalities seen in both the CA3 and dentate gyrus regions of the hippocampus are apparent throughout development and in adult animals. If the impairment of LTP were due to morphological abnormalities in the inputs to CA1, then a defect in LTP would be expected at all ages.

4.4.6. Genetic background effects

The results presented in this thesis demonstrate the effect of genetic background on the induction of LTP in the hippocampus of fyn knockout animals. The animals used in this study were from the C57BL/6 and 129/Sv inbred lines. The two strains of mice display very different patterns of behavior in many standard tests of cognition, which may have an electrophysiological basis. The reversal of the $fyn^{-/-}$ LTP phenotype between these two strains adds further evidence to this hypothesis. In this

thesis animals with a hybrid C57BL/6 x 129/Sv genetic background ($fyn^{-/-}$), showed no reduction in the level of LTP following tetanic stimulation (Figures 9, 10 and 11). In animals from an inbred 129/Sv strain a significant reduction in the level of LTP observed was apparent at two stimulation intensities ($fyn^{129/Sv-/-}$, Figures 15 and 16).

Embryonic stem cells (ES cells) derived from the 129 strain of animals are commonly used for gene ablation and targeting experiments (as was the case in this study, Stein et al. 1992, Simpson et al., 1997). The precise reason why this particular strain lends itself to this technique is not known, but ES cells from this lineage are stable in culture and provide competent cells for reintroduction to the mouse germline (Gerlai, 1996). 129 type ES cells carrying the targeted mutation are introduced into blastocyst stage embryos and the chimeric embryos allowed to develop to term. These mice are raised to adulthood, and crossed with 'wild type' mice to produce heterozygote offspring for the gene in question. These animals can then be backcrossed to provide homozygote, heterozygote and wild type offspring for use in experiments. If the genetic background of the ES cell line and the mice used to mate the chimeric animals produced is not identical, hybrid F_1 offspring animals with one set of chromosomes from the 129 strain and one set of chromosomes from the mating strain (e.g. C57BL/6) are produced. The recombination pattern between littermates may well be significantly different. This means that even littermate wild type animals do not constitute a suitable control group for the experimental animals in question. This can be addressed by either using a suitable breeding strategy (Banbury conference on genetic background in mice, 1997) or by maintaining the mutation to the same inbred strain throughout the genetic manipulation.

The $fyn^{-/-}$ hybrid mice used in this study were maintained by successively backcrossing animals on to a C57BL/6 background. C57BL/6 mice are active, and perform well in behavioral tasks. Comparatively, the 129/Sv strain of mice could be considered passive in behavioral tests (Gerlai, 1996). Due to accidental and deliberate outcrossing of this strain of mice, considerable genetic variation exists in substrains and ES cells derived from them (Simpson et al., 1997). This presents a

problem in mutant animals, as the flanking region surrounding the gene of interest will be from the 129 strain, whereas the same locus in wild type littermates will be from the C57BL/6 strain. If there are inherent mutations within this flanking region associated with the 129 strain used, then offspring will become homozygous for these mutations independently of the gene of interest. If any of these random mutations have position effects or directly regulate other genes important for the phenotype being studied, the results generated from hybrid animals must be taken with extreme caution (Lathe, 1996).

The use of transgenic technology to study the effects of loss of a particular gene in cognition has advanced dramatically over the last ten years. Molecular techniques have also advanced significantly over this time period and a large amount of data has implicated many molecules and receptors as regulators of higher order brain function (e.g. Silva et al., 1992, Zhuo et al., 1993, Huang et al., 1995, Aiba et al., 1994, Tsien et al., 1996, Jia et al., 1996, Sanes and Lichtman, 1999). However, the analysis of these data is extremely complex due to the number of variables involved. It is now becoming clear that genetic background factors can play a significant role in many behavioral test procedures, anatomy, response to pharmacological agents and certain evidence points to effects of genetic background on electrophysiological phenotypes (the data presented in this thesis adds weight to this argument and see below).

For example behaviorally C57BL/6 mice perform well in the Morris water maze test, showing a lower escape latency and greater learning over a three day trial period, a significantly reduced floating time and a higher locomotion rating as compared to 129/Sv mice (Wolfer et al., 1995). Upchurch and Wehner showed some time ago that the learning ability of different inbred strains varies considerably. In fact, certain strains, such as BALB, C3H and 129/SvJ lines are incapable of place learning (Upchurch and Wehner, 1988). DBA/2 mice display poor performance in the water maze that has been attributed to a protein kinase C deficiency present in this strain (Wehner et al., 1990). Also of note is the susceptibility of DBA/2 mice to audiogenic seizures to which C57BL/6 mice are resistant (see below, Seyfreid et al., 1978). The behavior of hybrid animals used in this study has been reported to be

normal in contradiction with the Grant et al. study (see below and Huerta et al., 1996). In a recent study of behavioral testing all experimental conditions were standardised as much as possible. Experiments were performed in three different laboratories and commenced on the same day, at the same time whilst controlling as many environmental factors as possible (e.g. age, sex, animal housing and the same brand of feed, light / dark cycle, breeding and genetic background). The results showed that several behaviors were influenced not only by the strain of animals used, but also on the particular laboratory site in which the animals were tested. The authors suggest that these environmental factors display such magnitude that subtle effects of a molecular ablation could well be overlooked (Crabbe et al., 1999).

Genetic background effects have also been noted in electrophysiological assay. For example in anaesthetized animals, a form of LTP in the dentate gyrus shows greater persistence in C57BL/6 mice compared to DBA/2 mice (Matsuyama et al., 1997). In a more recent study in awake freely moving animals, C57BL/6 mice show a longer lasting *in vivo* LTP in the dentate gyrus and a reduced threshold for population spike (PS) potentiation compared to DBA/2 mice. In line with the lower threshold for PS potentiation, the C57BL/6 mice alternate choices in a T maze behavioral test more than DBA/2 mice (Jones et al., 1999). 129 Ola mice show a significantly larger maximal EPSP slope and population spike amplitudes in the dentate gyrus compared to four other mouse strains (C57 albino, C3H, FVB/N and DBA/2). Paired pulse facilitation in contrast was similar across all strains, as was LTP induced in this area, with the exception of DBA/2 mice which show a deficit in maintenance of LTP (Bampton et al., 1999). Certain strains of mice are predisposed to epileptic activity, such as strain E1 (Rise et al., 1991) which could interfere with the interpretation of results generated in the hippocampus.

The differential response of inbred strains to many chemicals has been noted, for example markedly different sensitivities to ethanol, morphine, phencyclidine, cocaine and nicotine applications (Church and Feller, 1979, Brase et al., 1977, Miner and Collins, 1989, de Fiebre and Collins, 1993, Tolliver and Carney, 1994, Holsztynska et al., 1991). This may indicate that different strains of mice have

different biochemical leanings, perhaps in the levels of expression of certain enzymes. C57BL/6 mice show no hippocampal neuronal cell death or evidence of neuronal cell damage following kainate injections in contrast to rat strains and other mouse strains (FVB/N and 129/SvEMS, Schauwecker and Steward, 1997). Mice lacking in the p53 tumor-suppressor gene, derived from 129/Sv ES cells and mated to a C57BL/6 line, showed no selective hippocampal neuronal loss following kainate injections, implicating p53 in a neuroprotective mechanism. Examination of another line of $p53^{-/-}$ mice, derived from a 129/SvEMS background, did not show this neuroprotection, indicating that the effect of p53 deletion is background specific. The C57BL/6 alleles produce this neuroprotective mechanism themselves; irrespective of the deletion involved (Choi, 1997). In a study of epidermal growth factor receptor deletion mutants, a more benign phenotype was seen in 129/Sv x C57BL/6 background than in a 129/Sv background (Pollard et al., 1994).

Anatomical differences between strains have also been observed. The development of the corpus callosum and the hippocampus is different between mouse strains and dysgenesis of the corpus callosum is one of the major abnormalities of the inbred 129 strain (Wolfer et al., 1995). Projections of the mossy fibre pathway, in particular the intra / infrapyramidal mossy fibre projection in area CA3, have been shown to be significantly different in DBA/2 and C3H lines of mice which has been linked to differing performances in a two way avoidance behavioral paradigm (Lipp et al., 1989).

All of these examples indicate that the specific background, resulting from the strain of ES cells used to generate chimeric animals through to the strain of mice used to maintain any colony, can influence the phenotypes studied. It will become clearer as more examples arise that the neuroscience community as a whole will have to pay closer attention to the strategies used to generate genetic mutants for the study of cognition. It is, however, worthwhile noting that the use of different strains, be they inbred or hybrid animals, can also be beneficial in the understanding of how cognitive processes function. If a phenotype is apparent on one background, and is

lost on another, analysis of the differences between these strains could provide invaluable information itself.

Another major consideration in animals generated in a hybrid background is the role of the flanking region surrounding the gene of interest. Eliminating the 129-type genes that surround the locus of the gene of interest would require a large number of backcrossings. It has been estimated that the length of the 129-type chromosome segment introduced into C57BL/6 genome would be 16 centimorgans (cM) after 12 backcrosses (2 years of breeding, Festing, 1992). This represents around 1% of the entire mouse genome, containing some 300 genes. Linked genes or those with position effects could play a significant role in behaviors, and as hybrid mice will become homozygous for some of these flanking genes through successive backcrosses, these could lead to major effects on phenotypes in offspring generated from higher order generations. The hybrid $fyn^{-/-}$ mice used in this study were backcrossed an unknown number of times. Genetic analysis of the loci surrounding the fyn gene could indicate which alleles had become homozygous in animals which did not display the reduction in LTP and provide clues as to any linked genes which may influence the role of fyn in synaptic plasticity mechanisms. This analysis was beyond the scope of this project but could be a future line of interest for these animals.

4.4.7. Additional behavioral data on $fyn^{-/-}$ mice

Spatial learning in the Morris water maze was reported to be impaired in $fyn^{-/-}$ mice (Grant et al., 1992). The mice used for this study were 7-9 weeks old and from an inbred 129/Sv line. In light of the publication of Kojima et al. (1997) showing that the reduction of LTP in $fyn^{-/-}$ mice is not apparent until the 14 week of age, this raises questions as to the link between spatial learning and hippocampal LTP implied in the Grant et al. paper. If no LTP deficit is apparent in animals of this age, then the spatial learning deficit seen can not be attributed to this change in electrophysiological phenotype.

Huerta et al. (1996) performed spatial learning tests on animals from the same hybrid background as the Edinburgh $fyn^{-/-}$ colony. This study reports that, unlike animals in the Grant et al. study, $fyn^{-/-}$ mice were able to learn the position of a hidden platform over several blocks of trials. A significantly greater escape latency was found in the $fyn^{-/-}$ mice, which improved with training. In comparison, wild type (inbred C57BL/6J) mice displayed a similar improvement in escape latency with training, but displayed a significantly lower escape latencies on all trial blocks. Huerta et al. explains the increased escape latency of the $fyn^{-/-}$ mice, not through an inability to learn spatial information, but due to abnormal swimming behavior. $Fyn^{-/-}$ mice 'float' when initially introduced to the water maze, i.e. the animals stay in one location and show no locomotor activity. This behavior indicated an increased fearfulness in these mice. The authors of this study stimulated a swimming response by touching the mice on the hindquarters, and found that although the mice displayed uncoordinated swimming behavior they were able to locate the hidden platform well. Wild type mice showed no difference in ability when also stimulated in the same manner. Direct evidence that the $fyn^{-/-}$ mice do learn the spatial information correctly comes from the transfer test. Both wild type mice and $fyn^{-/-}$ mice spent a significantly greater amount of time than chance searching the correct quadrant and significantly less time than chance in the opposite quadrant. Both sets of mice performed equally well in the visible platform version of this test. The results are not in agreement with the Grant et al study. Several differences exist in the training protocols used in between the studies, and in the sequence of tests applied. The training protocol used in the Huerta study follows established standards for assessing the spatial abilities of rodents in the water maze, whereas the Grant et al. study used a less intense protocol.

The data presented in this thesis provides another possible explanation. Spatial learning in the water maze has been shown to be dependent on normal hippocampal function (Morris, 1982, 1989) and upon the activation of the NMDA receptor system (Morris et al., 1986). Hippocampal LTP has been postulated as the cellular basis for this form of spatial learning. The lack of reduction of LTP presented in this thesis in

animals from the same background of mice used for the Huerta et al. 1996 study may indicate that this reversal of behavioral phenotype is linked to the genetic background modifications, and not to the actual fyn mutation itself. It should be noted however that the wild type control group used was a pure strain of C57BL/6J mice, and not littermates of the $fyn^{-/-}$ animals.

Cerebellar anatomy and motor function has been studied in $fyn^{-/-}$ mice and no apparent defects were seen (Seth Grant - Personal communication). Motor function was studied in $fyn^{129/Sv-/-}$ mice (in a pure 129/Sv genetic background, Cain et al., 1995) and normal motor behaviors were observed, again implicating genetic background modifications in the abnormal swimming behavior seen in the Huerta et al. study. The abnormal 'floating' behavior may, however, be linked to reports of increased fearfulness in mice with a targeted mutation in the fyn locus (Miyakawa et al., 1994, Miyakawa et al., 1995, Miyakawa et al., 1996).

4.4.8. Other studies in fyn knockout mice

There are two distinct forms of fyn knockout mice, one which was created by replacing the first coding exon of the fyn gene with a neo expression cassette ($fyn^{-/-}$, Stein et al., 1992). The other fyn knockout mice was independently derived by inserting the β-galactosidase gene (*lacZ*) into the fyn gene locus (fyn^{Z} / fyn^{Z}, Yagi et al., 1993). The animals used in the Grant et al. (1992), Huerta et al. (1996) and this study are mice derived by Stein et al. Details of other relevant phenotypes are detailed below.

Behaviorally fyn^{Z} / fyn^{Z} mice display increased fearfulness in the light-dark choice test and novelty preference and passive avoidance tests (Miyakawa et al., 1994). This may explain the 'floating' syndrome reported by Huerta et al. (1996) in the Morris water maze. Mice are described as floating in the initial point of entry to the pool, which is reminiscent of the freezing response seen in animals to aversive stimuli (i.e. fear). Fear induced behaviors are linked to the amygdala and this

structure also expresses high levels of fyn transcripts (Yagi et al., 1993). Fyn^Z / fyn^Z mice also show an enhanced susceptibility of audiogenic seizures that are more likely to develop into the stronger seizure syndrome clonus (Miyakawa et al., 1995). Further evidence for the increased timidity of these animals was detailed by the same group in the radial arm maze, open-field and elevated plus maze behavioral tasks. Fyn^Z / fyn^Z mice appeared to learn the spatial aspects of these tasks to the same levels as heterozygous controls ($+ / fyn^Z$). The authors postulate that the behavior seen in the Morris water maze test by Grant et al. may be due their increased fearfulness and not from impaired spatial learning as the repeated placement of animals in water could be considered a stressful and aversive task (Miyakawa et al., 1996).

Drug-induced seizures protocols have also been tested on this line of fyn knockout mice. Fyn^Z / fyn^Z mice showed increased susceptibility to myoclonic seizures as compared to $+ / fyn^Z$ littermates upon acute systemic administration of pentylenetetrazol, picrotoxin, bicuculline (all GABA-ergic antagonists), kainic acid and NMDA (Miyakawa et al., 1996). A study of GABA-ergic signalling in mice overexpressing either wild type fyn (w-fyn) or a mutant constitutively active form of fyn (m-fyn) indicates a significant role for fyn in the regulation of GABA-ergic function. Application of the $GABA_A$ receptor antagonist, bicuculline, to wild type and w-fyn slices enhanced fEPSPs by approximately 150% on average, whilst m-fyn slices were only enhanced by 41% on average. This indicated that GABA-ergic inhibition is reduced in m-fyn mice (Lu et al., 1999). GABA receptors can be tyrosine phosphorylated and $GABA_A$ receptor mediated currents in cultured and hippocampal neurones have been shown to be regulated by tyrosine phosphorylation (Moss et al., 1995, Herron and Grant, 1997). The increased basal transmission and reduced GABA-ergic function may well contribute to the epileptogenic abnormalities in mice lacking fyn kinase (Cain et al., 1995, Kojima et al., 1999).

This may also relate to LTP as the integration of glutaminergic and GABA-ergic systems sets the probability level for inducing LTP. Homozygous fyn mice from the colony used in the Kojima et al. (1997) fyn rescue study were prone to severe seizures which often led to death. These mice also displayed a classic hydrocephaly,

in which the bones of the skull were distorted to form a 'cone' shape, a large fluid filled cavity was seen in the posterior brain cavity and the brain tissue itself was soft and 'watery'. This was only seen in homozygous offspring and the numbers of mice displaying this abnormality increased with backcrossing. Around 10 - 15% of all homozygous fyn offspring were found to have this hydrocephaly. In one case where homozygous fyn mice were backcrossed, all offspring displayed this phenotype (Isabelle Mansuy – Personal communication). No known cases of seizures leading to death were found in the $fyn^{-/-}$ mice from the Edinburgh colony, however several mice did displayed the hydrocephaly described above, but only in the C57BL/6 x 129/Sv hybrid background. Unfortunately, no data was obtained regarding this apparently new phenotype which could indicate the role of fyn tyrosine kinase in a neurodegenerative process.

Investigations into the role of fyn in the induction of LTP have been developed further recently by two studies (Lu et al., 1999 and Narisawa-Saito et al., 1999). The former paper details studies of mice overexpressing either a mutant constitutively active form of fyn (m-fyn) or wild type fyn (w-fyn). These transgenes were driven by a CaMKII promoter that turned on expression late in neural development, avoiding the disruption of hippocampal architecture seen in mice lacking fyn from birth. In these animals a theta burst protocol (four pulses at 100Hz) was used to induce LTP. As fyn has been reported to affect the threshold for induction, both weak (2 bursts separated by 200ms) and strong (either 5 or 10 bursts separated by 200ms) induction protocols were used. In animals expressing w-fyn or wild type controls the weak stimuli produced a short-term potentiation which decayed to baseline within 60 minutes. In mice expressing the m-fyn transgene however, this protocol produced LTP (143% of baseline at 60 minutes). The stronger induction protocols induced LTP in wild type, w-fyn and m-fyn expressing mice. This evidence further implicates fyn in the modulation of the level of LTP induction, but provides no new evidence as to the mechanism of this effect.

The role of fyn in the signal transduction of brain derived neurotrophic factor (BDNF) and platelet derived growth factor (PDGF) receptor activation has recently

been reported (Narisawa-Saito et al., 1999). BDNF can modulate NMDA receptor channel activity, calcium channels (Suen et al., 1997, Lesser et al., 1997) and can modulate synaptic transmission as well as LTP (Levine et al. 1995, Korte et al., 1996, Patterson et al., 1996). Both BDNF and PDGF signalling pathways are associated with src family protein tyrosine kinases (Kremer et al., 1991, Iwasaki et al., 1998, Kypta et al., 1990). Narisawa-Saito et al. show that the expression of AMPA receptor subunits GluR1, GluR2 and GluR3 is enhanced by BDNF and PDGF in a fyn dependent manner using a novel specific inhibitor, PP1 (Hanke et al, 1996). In line with this evidence, BDNF application to primary neuronal cultures showed enhancement of AMPA receptor subunits in wild type cells (C57BL/6) but not in fyn^{Z} / fyn^{Z} cells with no change in the level of NMDA receptor subunit proteins. The level of AMPA receptor subunit proteins was also established *in vivo*, $fyn^{-/-}$ mice (Stein animals as used in this thesis) showed significantly reduced protein levels of AMPA receptor subunits GluR1, GluR2 and GluR3, whereas mRNA levels were not affected. This has important implications for the induction of LTP in fyn knockout mice. If the number of AMPA receptors expressed at the postsynaptic site is reduced, then the level of depolarisation produced by high frequency stimulation could be lowered, attenuating the activation of NMDA receptors, and thus reducing the subsequent calcium influx. This will reduce the level of potentiation seen in these animals, and may explain the need for a higher level of stimulation to induce robust LTP. Src levels were also upregulated in these $fyn^{-/-}$ mice, and those animals that displayed higher levels of AMPA receptor subunits were correlated with those mice that displayed the highest levels of src. It appears that src compensation for fyn does occur, and that this can also modulate the expression level of AMPA receptor proteins (Narisawa-Saito et al., 1999).

4.5. Summary

Fyn tyrosine kinase is important for the regulation of synaptic transmission in area CA1 of the hippocampus. Fyn is involved in the modulation of the three main receptor subtypes, the NMDA receptor, the AMPA receptor and the GABA receptor involved in regulating transmission at this synapse. The mechanism by which this important kinase mediates all of these effects however is still unclear. This is mainly due to the lack of known fyn specific substrates. The development of specific pharmacological agents against fyn would allow a more thorough investigation into the pathways which mediate these effects. Further study into the control of other forms of synaptic plasticity, such as short-term potentiation and post-tetanic potentiation may help uncover some of these mechanisms. Due to the clear role of fyn in organising the architecture of the dentate gyrus, a study of LTP in this structure may also help elucidate mechanisms of action, as NMDA receptor independent LTP is also found in this region, and may allow the separation of some of the multiple roles fyn obviously plays. The intracellular injection and / or extracellular application of the novel fyn specific inhibitor PP2, through patch clamp recordings would also be a good line of experimentation. In the next chapter, the involvement of fyn in the short-term plastic phenomenon of paired pulse facilitation is investigated further.

Chapter V

Results

Short term plasticity

5.1. $Fyn^{-/-}$ paired pulse facilitation experiments

Basal synaptic transmission was established to be normal in the Grant et al. paper by recording PPF at one inter stimulus interval (ISI) of 50ms. The profile of PPF facilitation over a wide range of ISIs was performed. Examples of an individual experiment for both $fyn^{-/-}$ and wild type mice are shown in Figures 19B (wild type hybrid) and 19C ($fyn^{-/-}$). The averaged profile of PPF in wild type mice (n = 25) and $fyn^{-/-}$ mice (n = 18) is shown in Figure 19A (see Table 7). The peak of facilitation was seen at the 50ms inter-stimulus interval and the level of facilitation decreased as the inter-stimulus interval increased. A statistical difference was achieved between the $fyn^{-/-}$ and wild type groups using a one way ANOVA test ($P < 0.001$, $F = 13.764$), indicating that there is a clear difference in the population means.

5.2. $Fyn^{129/Sv-/-}$ paired pulse facilitation experiments

The extracellular profile of paired pulse facilitation was also studied in the pure 129/Sv inbred background. Examples of individual experiments are displayed in Figures 20B (wild type 129/Sv) and 20C ($fyn^{129/Sv-/-}$). The averaged profile of PPF in wild type 129/Sv mice (n = 25) and $fyn^{129/Sv-/-}$ mice (n = 23) is shown in Figure 20A (see Table 8). The effect of genotype was tested using a one way ANOVA, which demonstrates a statistical difference between the $fyn^{129/Sv-/-}$ population and wild type population ($P < 0.001$, $F = 35.955$).

5.3. Tyrosine kinase inhibitors and paired pulse facilitation

To determine if the effects on PPF seen in the $fyn^{129/Sv-/-}$ mice were specifically due to the lack of functional fyn kinase, a specific inhibitor, PP2, was applied to wild type 129/Sv slices (n = 10). PP2 (100 nM) caused a reduction in the averaged ratio at all ISIs (Figure 21A, Table 9) and a clear reduction in the fEPSP slope of both the conditioning (1^{st}) and test (2^{nd}) responses. The use of an inactive structural analogue,

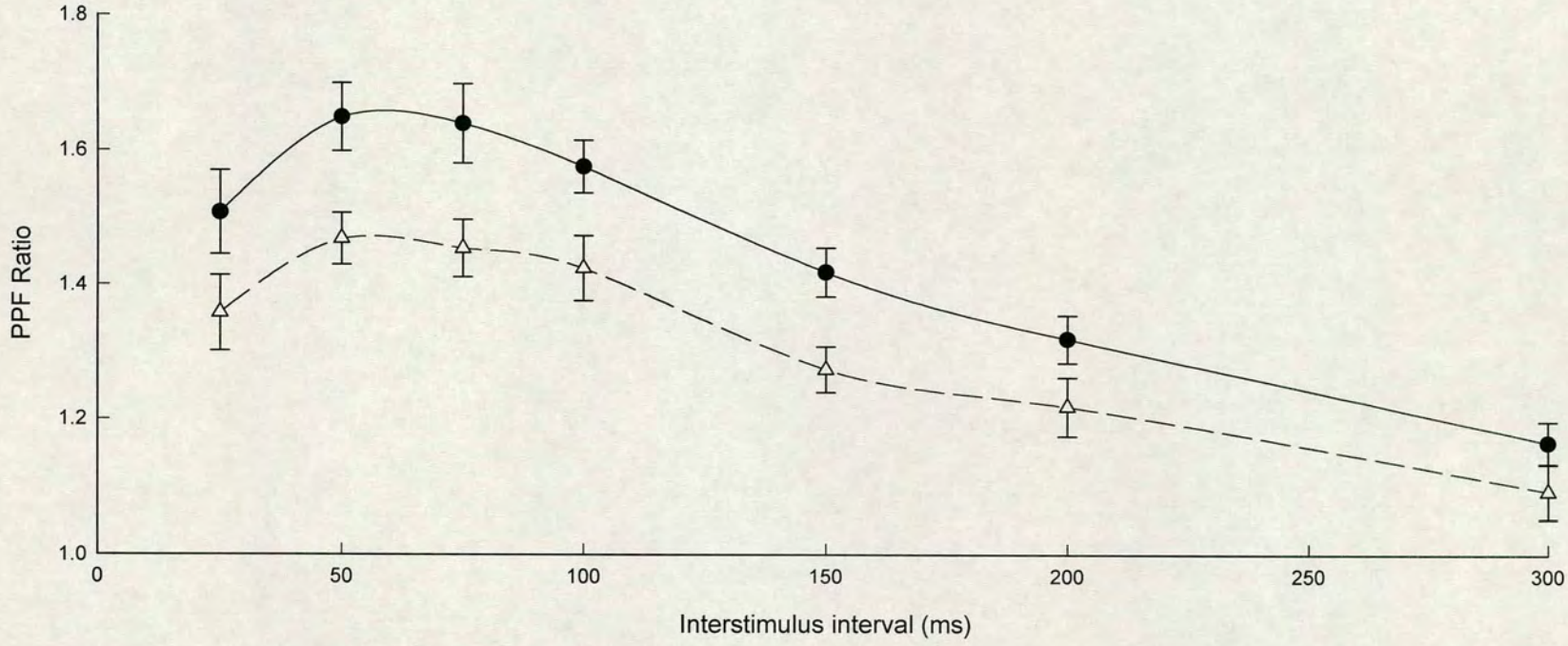
Figure 19 **Extracellular paired pulse facilitation experiments in the hybrid (C57BL6 x 129/Sv) genetic background (See 5.1.)**

A The averaged profile of PPF over the inter-stimulus range of 25-300ms in both $fyn^{-/-}$ (white triangles) and wild type hybrid animals (black circles). Error bars indicate the mean ± standard error.

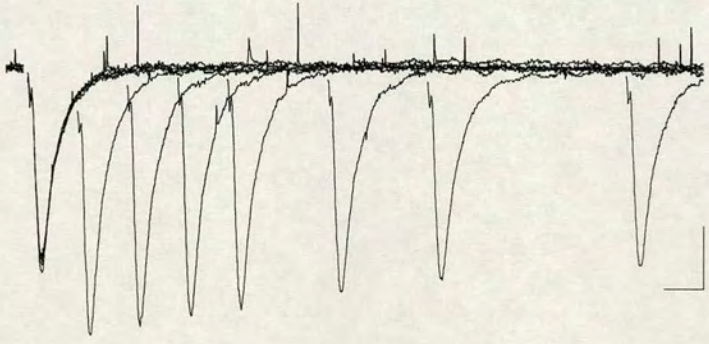
B Example fEPSPs at all inter-stimulus intervals in wild type hybrid animal, Scale bars, 0.2mV, 20ms

C As above for $fyn^{-/-}$

A


PPF Ratio
1.8
1.6
1.4
1.2
1.0
0
50
100
150
200
250
300
Interstimulus interval (ms)

B



C

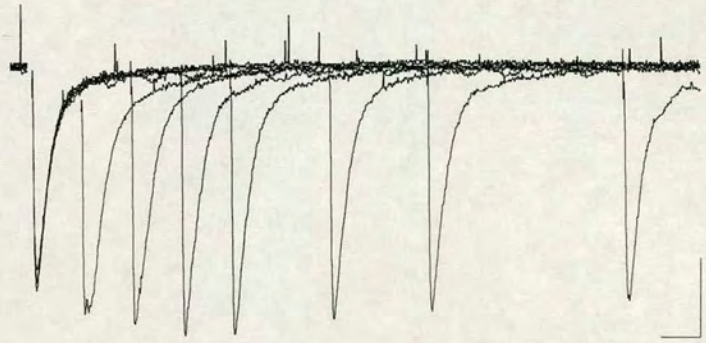


Table 7 – PPF ratios in $fyn^{-/-}$ and wild type hybrid animals

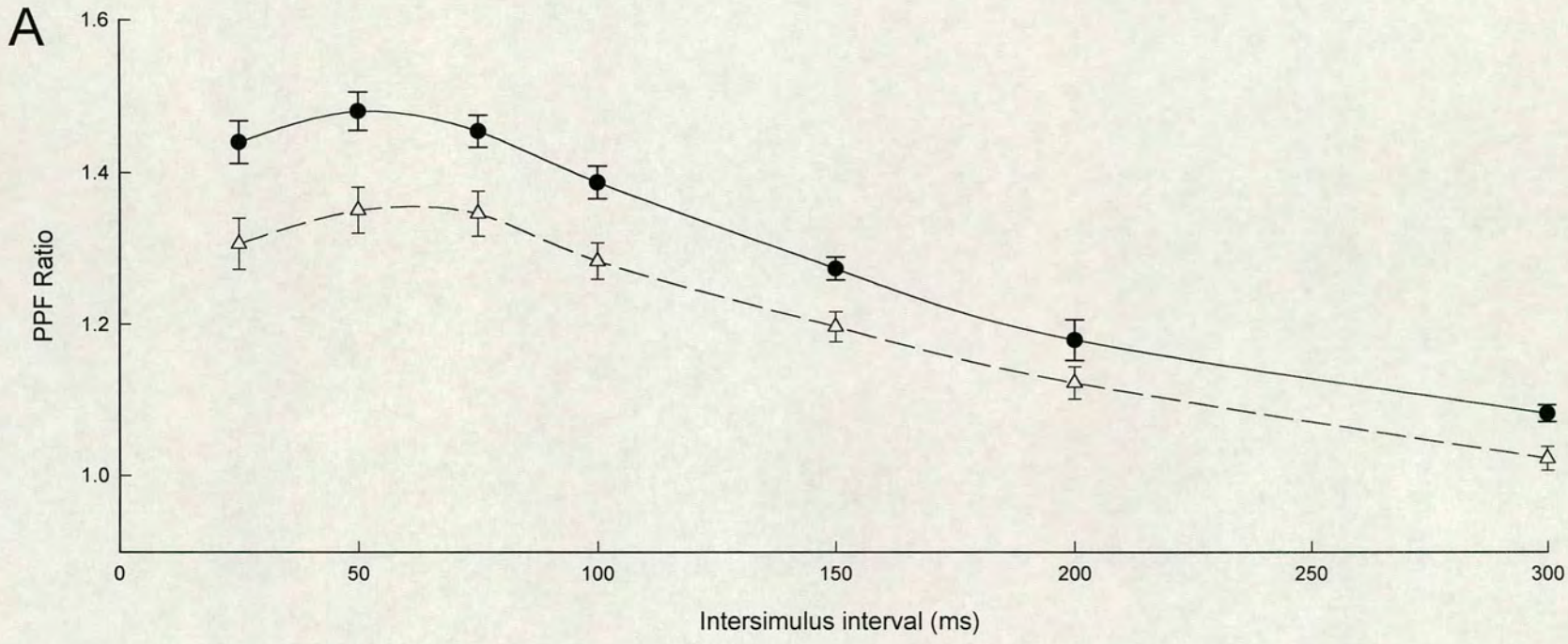
Interval (ms)	*Wild Type PPF Ratio*	*Wild Type S.E.M.*	*Fyn PPF Ratio*	*Fyn S.E.M.*
25	1.507	0.062	1.358	0.055
50	1.648	0.050	1.468	0.038
75	1.639	0.058	1.417	0.045
100	1.575	0.039	1.424	0.048
150	1.418	0.036	1.274	0.033
200	1.319	0.035	1.218	0.043
300	1.166	0.030	1.093	0.040

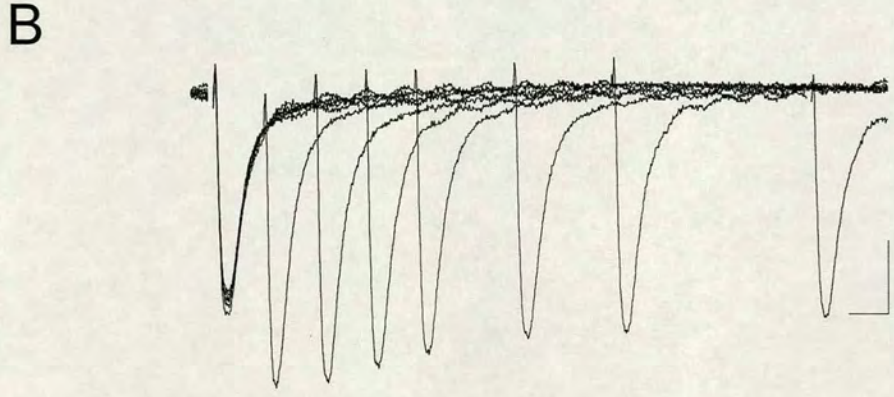
Figure 20 **Extracellular paired pulse facilitation in the 129/Sv inbred genetic background (See5.2.)**

A The averaged profile of PPF over the inter-stimulus range of 25-300ms in both $fyn^{129/Sv-/-}$ (white triangles) and wild type 129/Sv animals (black circles). Error bars indicate the mean ± standard error.

B Example fEPSPs at all inter-stimulus intervals for wild type 129/Sv animal, Scale bars, 0.2mV, 20ms.

C As above for $fyn^{129/Sv-/-}$.


A
PPF Ratio
1.6
1.4
1.2
1.0
0
50
100
150
200
250
300
Intersimulus interval (ms)


B

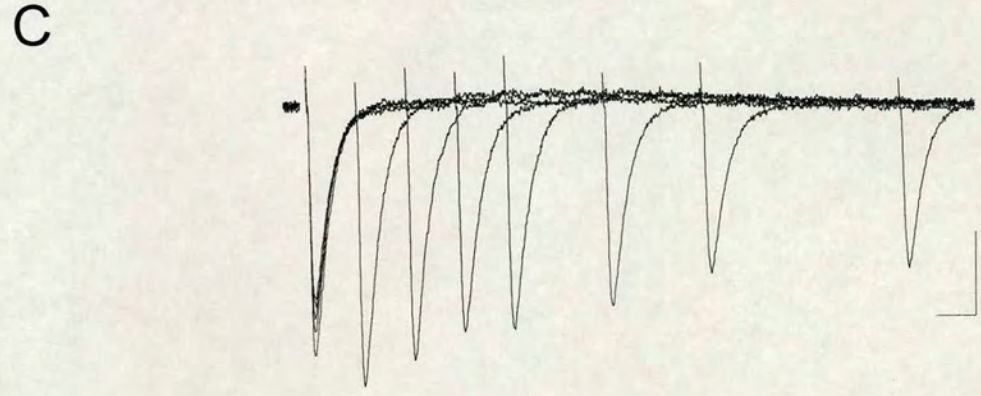

C

Table 8 – PPF ratios in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

Interval (ms)	*Wild Type PPF Ratio*	*Wild Type S.E.M.*	*Fyn PPF Ratio*	*Fyn S.E.M.*
25	1.440	0.028	1.306	0.034
50	1.480	0.025	1.351	0.030
75	1.454	0.021	1.346	0.029
100	1.387	0.021	1.284	0.024
150	1.273	0.015	1.197	0.019
200	1.179	0.027	1.122	0.021
300	1.082	0.011	1.022	0.016

PP3 (n = 10), gave a profile of PPF very similar to that seen in control medium (n = 12, Figure 21B, Table 10). A one way ANOVA test was used to test the population means from the control group and the PP2 treated group. This showed a statistical difference between the population means ($P < 0.001$, $F = 30.138$).

5.4. Developmental effects on paired pulse facilitation

Animals used in all studies presented so far were greater than 14 weeks of age (see Kojima et al., 1997, Chapter 4). To determine if the effects seen in PPF were also age dependent in these mice i.e. dependent upon developmental changes, the PPF profile (25 – 300 ms inter-stimulus intervals) was determined in animals less than 6 weeks old. The results are shown in Figure 22A and Table 11. The trend of $fyn^{129/Sv-/-}$ mice displaying a lower facilitation ratio is apparent. These data can be compared with the data sets generated in animals over 14 weeks of age (Figures 22B and 22C). In both wild type 129/Sv mice (n = 14) and $fyn^{129/Sv-/-}$ mice (n = 11), the averaged ratio seen was higher in the younger animals (see Table 12, 13). A one way ANOVA was used to compare the effect of genotype, and showed a statistical differences between the population means at this age (6wks, $P < 0.001$, $F = 18.305$). A significant difference also exists between the young animals and old animals in both $fyn^{129/Sv-/-}$ mice and wild type 129/Sv mice ($P < 0.001$, $F = 16.391$, $P < 0.001$, $F = 43.263$ respectively).

5.5. Intracellular recordings of paired pulse facilitation

Intracellular sharp electrode recording of the profile of AMPA receptor mediated PPF at ISIs of 50, 75, 100, 150, 200 and 300 ms in CA1 pyramidal cells from $fyn^{129/Sv-/-}$ (n = 8) and wild type 129/Sv (n = 9) hippocampal slices is shown in Figure 23 (see 2.9.2., for the pharmacological isolation of AMPA receptor mediated responses). At all inter-stimulus intervals, $fyn^{129/Sv-/-}$ cells display a smaller facilitation ratio than seen in wild type 129/Sv cells (Table 14). A one way ANOVA

Figure 21 **The effects of a tyrosine kinase inhibitor and its inactive structural analogue on extracellular PPF in wild type 129/Sv animals of an inbred genetic background (See 5.3.)**

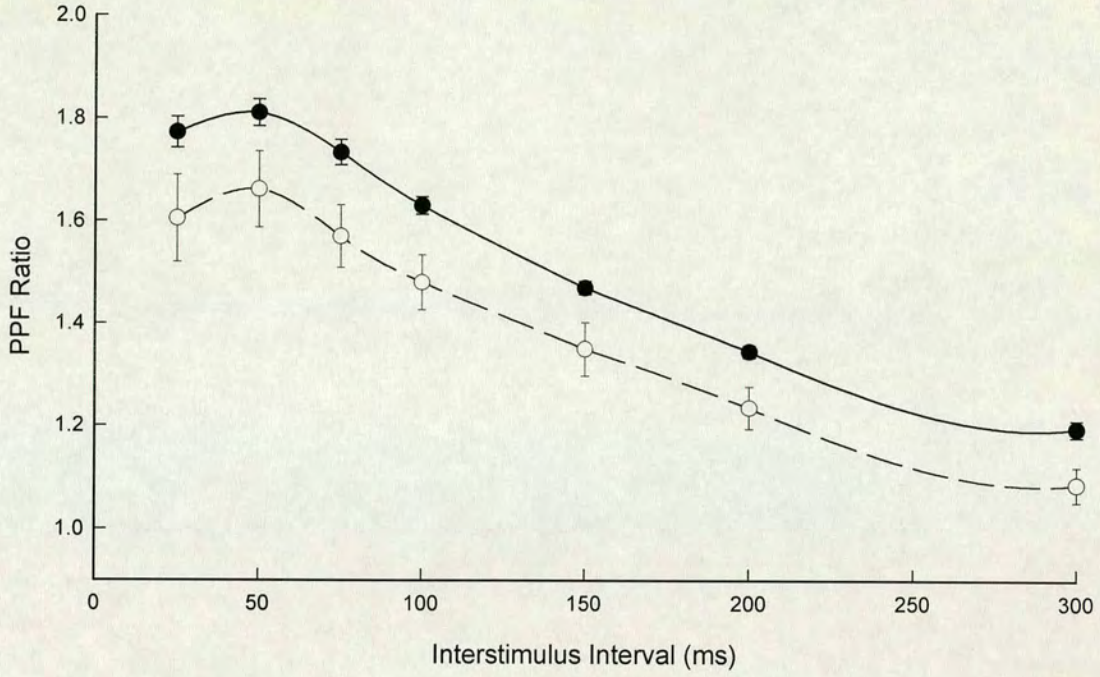
A The effects of extracellular administration of PP2 (100nM) on wild type 129/Sv slices. A significant reduction was seen of the control response (black circles) upon PP2 administration (white circles).

B The effects of PP3, an inactive analogue of PP2. No effect of PP3 (100nM, white triangles) was seen compared to the control response (black circles).

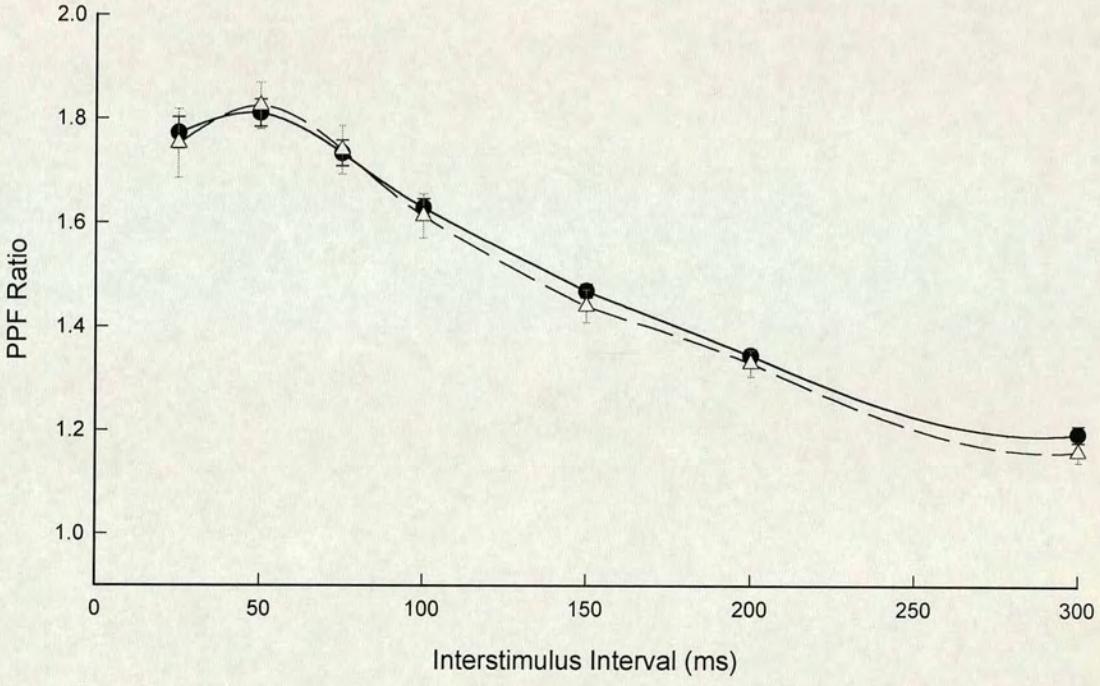
C Example fEPSP traces of the reduction in fEPSP size and slope brought about by PP2. Scale bar, 0.25mV, 10ms.

Error bars indicate the mean ± standard error.

A


2.0
1.8
1.6
1.4
1.2
1.0
PPF Ratio
0
50
100
150
200
250
300
Interstimulus Interval (ms)

B


2.0
1.8
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PPF Ratio
0
50
100
150
200
250
300
Interstimulus Interval (ms)

C

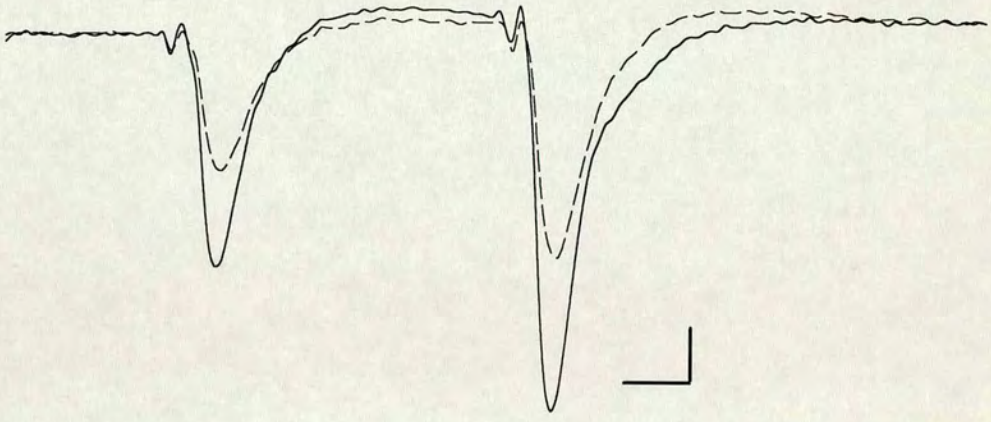


Table 9 – The effects of the tyrosine kinase inhibitor PP2 on PPF in wild type 129/Sv animals

Interval (ms)	*Control PPF Ratio*	*Control S.E.M.*	*PP2 PPF Ratio*	*PP2 S.E.M.*
25	1.772	0.030	1.604	0.084
50	1.810	0.025	1.661	0.074
75	1.733	0.024	1.569	0.061
100	1.628	0.017	1.479	0.053
150	1.469	0.013	1.349	0.052
200	1.345	0.011	1.235	0.041
300	1.194	0.016	1.086	0.033

Table 10 – The effects of the inactive structural analogue of PP2, PP3 on PPF in wild type 129/Sv animals

Interval (ms)	*Control PPF Ratio*	*Control S.E.M.*	*PP3 PPF Ratio*	*PP3 S.E.M.*
25	1.772	0.030	1.752	0.065
50	1.810	0.025	1.825	0.044
75	1.733	0.024	1.740	0.046
100	1.628	0.017	1.613	0.042
150	1.469	0.013	1.440	0.031
200	1.345	0.011	1.332	0.025
300	1.194	0.016	1.161	0.020

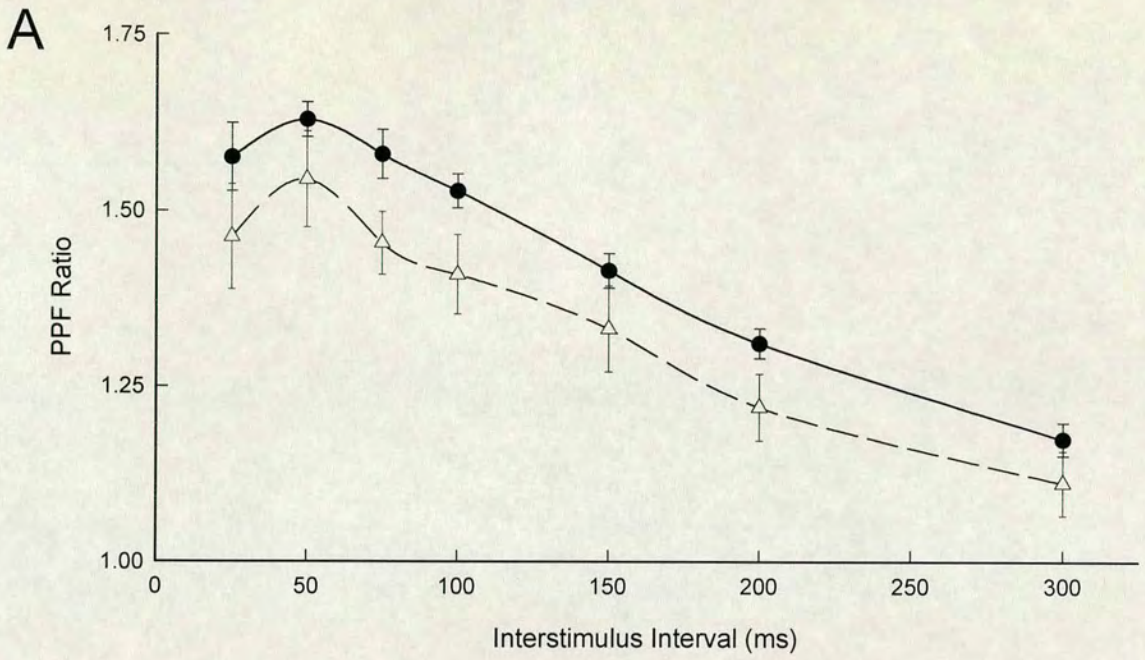
Figure 22 **Age dependent effects on extracellular PPF in 129/Sv animals (See 5.4.)**

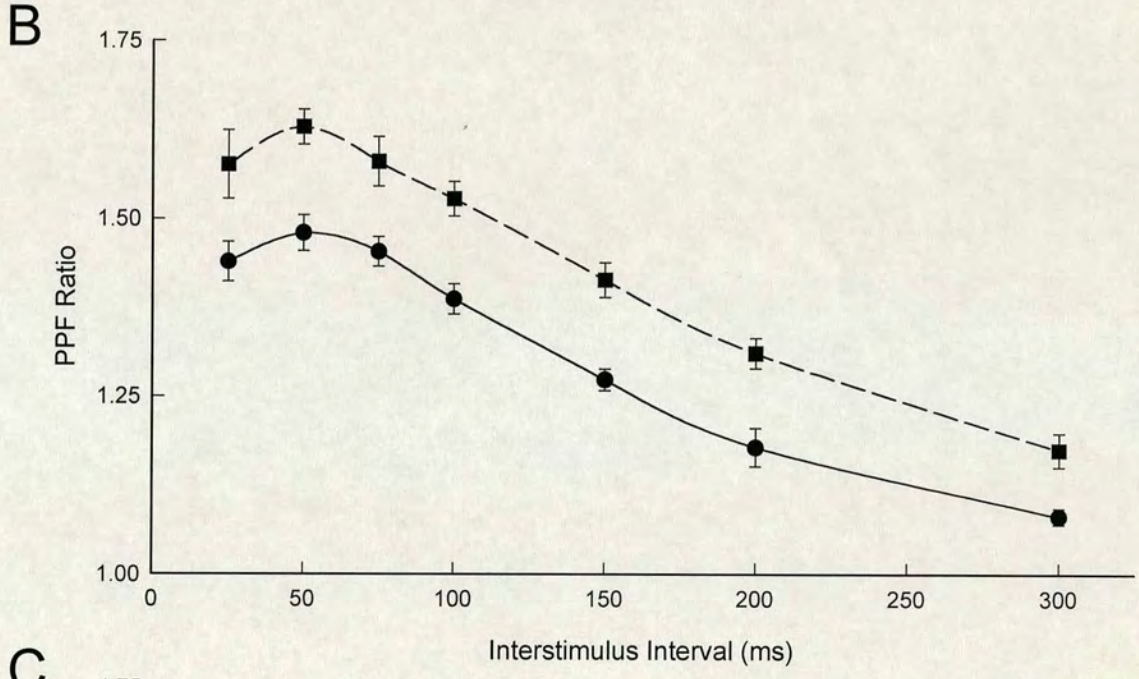
A The profile of PPF in animals under 6 weeks of age, $fyn^{129/Sv-/-}$ animals (white triangles) show a decreased level of facilitation compared to wild type animals (black circles)

B The comparison of young (black squares) and old (black circles) wild type 129/Sv animals.

C The comparison of young (white diamonds) and old (white triangles) $fyn^{129/Sv-/-}$ animals.

Error bars indicate the mean ± standard error.


A
1.75
1.50
1.25
1.00
PPF Ratio
0
50
100
150
200
250
300
Interstimulus Interval (ms)


B
1.75
1.50
1.25
1.00
PPF Ratio
0
50
100
150
200
250
300
Interstimulus Interval (ms)

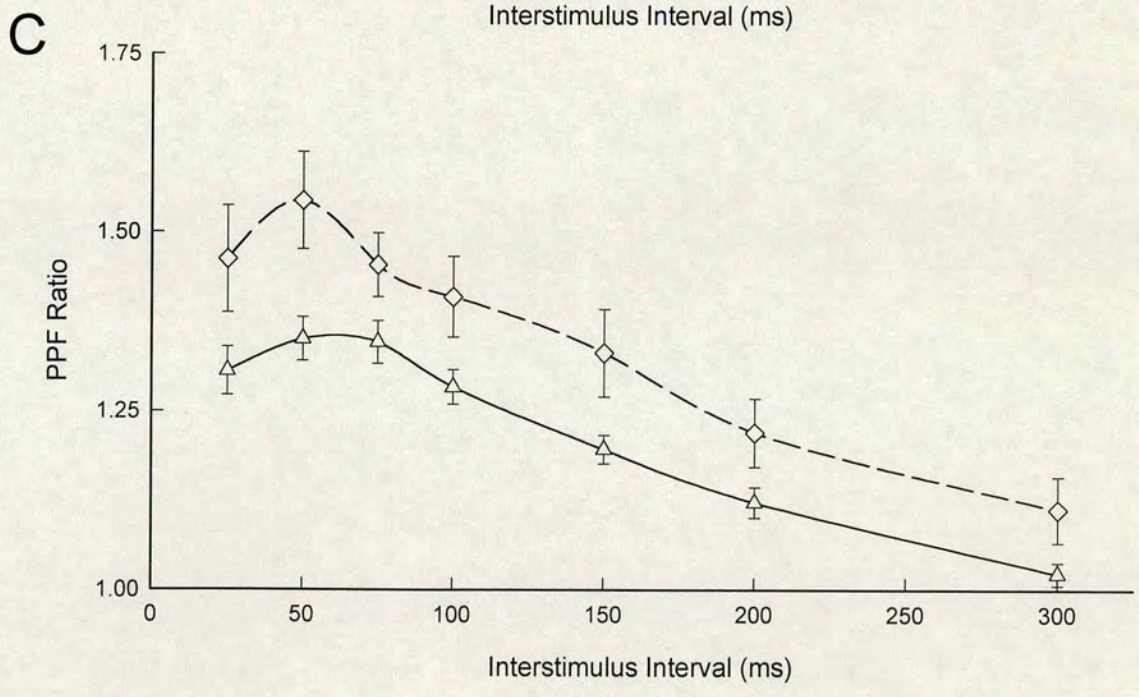

C
1.75
1.50
1.25
1.00
PPF Ratio
0
50
100
150
200
250
300
Interstimulus Interval (ms)

Table 11 – PPF in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals under 6 weeks of age

Interval (ms)	*Wild Type PPF Ratio (Young)*	*Wild Type S.E.M. (Young)*	*Fyn PPF Ratio (Young)*	*Fyn S.E.M. (Young)*
25	1.576	0.048	1.462	0.074
50	1.629	0.024	1.544	0.068
75	1.580	0.034	1.453	0.044
100	1.528	0.024	1.408	0.056
150	1.414	0.024	1.331	0.060
200	1.311	0.021	1.219	0.047
300	1.174	0.023	1.111	0.045

Table 12 – PPF ratios in old (>14 weeks) and young (<6 weeks) wild type 129/Sv animals

Interval (ms)	*Wild Type PPF Ratio (Old)*	*Wild Type S.E.M. (Old)*	*Wild Type PPF Ratio (Young)*	*Wild Type S.E.M. (Young)*
25	1.439	0.027	1.576	0.048
50	1.480	0.025	1.629	0.024
75	1.454	0.020	1.580	0.034
100	1.387	0.021	1.528	0.024
150	1.273	0.015	1.414	0.024
200	1.178	0.027	1.311	0.021
300	1.082	0.011	1.174	0.023

Table 13 – PPF ratios in old (>14 weeks) and young (<6 weeks) $fyn^{129/Sv-/-}$ animals

Interval (ms)	*Fyn PPF Ratio (Old)*	*Fyn S.E.M. (Old)*	*Fyn PPF Ratio (Young)*	*Fyn S.E.M. (Young)*
25	1.306	0.033	1.462	0.074
50	1.350	0.030	1.544	0.068
75	1.346	0.029	1.453	0.044
100	1.283	0.024	1.408	0.056
150	1.196	0.019	1.331	0.060
200	1.122	0.021	1.219	0.047
300	1.022	0.015	1.111	0.045

test on the population means demonstrates that there is a statistically different effect of genotype ($P < 0.0173$, $F = 2.287$) in these groups also. No data was recorded for the 25ms interval due to the overlap of AMPA receptor mediated EPSPs at this short latency (to avoid possible distortions to the initial slope measurements). Also depolarisation will reduce the AMPA receptor mediated EPSP as the driving force for the response will be reduced as the EPSP is activated closer to its reversal potential.

5.6. Modulation of presynaptic release by calcium

In wild type 129/Sv and $fyn^{129/Sv-/-}$ mice, reducing the extracellular calcium concentration to 1.3 mM (and raising the magnesium ion concentration to 2.5 mM) caused a decrease in the fEPSP size, and increased the PPF ratio.

Conversely, raising the extracellular calcium concentration to 4.5 mM (and reducing the magnesium ion concentration to 0.5 mM) caused an increase in the fEPSP size and a subsequent reduction in PPF ratio (see Table 1 for ACSF composition). The profile of paired pulse facilitation in these conditions is shown in Figures 24A and 24C. In 129/Sv wild type mice ($n = 8$) and $fyn^{129/Sv-/-}$ mice ($n = 7$), a significant effect of both low and high calcium containing solutions was seen (see Table 15, 16, 17 and 18). The changes in ratio from low / normal / high calcium containing solutions can be expressed as a percentage relative to the control response at each ISI and is shown in Figure 25. There was a trend for the $fyn^{129/Sv-/-}$ mice to show a smaller percentage change than wild type 129/Sv mice in all cases (see Tables 19 and 20). A one way ANOVA test at the low, normal and high calcium levels all showed a significant effect of genotype ($P < 0.001$, $F = 14.843$, $F = 14.086$ and $F = 5.975$ respectively).

Figure 23 **Intracellular recordings of paired pulse facilitation of the AMPA receptor mediated EPSP (See 5.5.)**

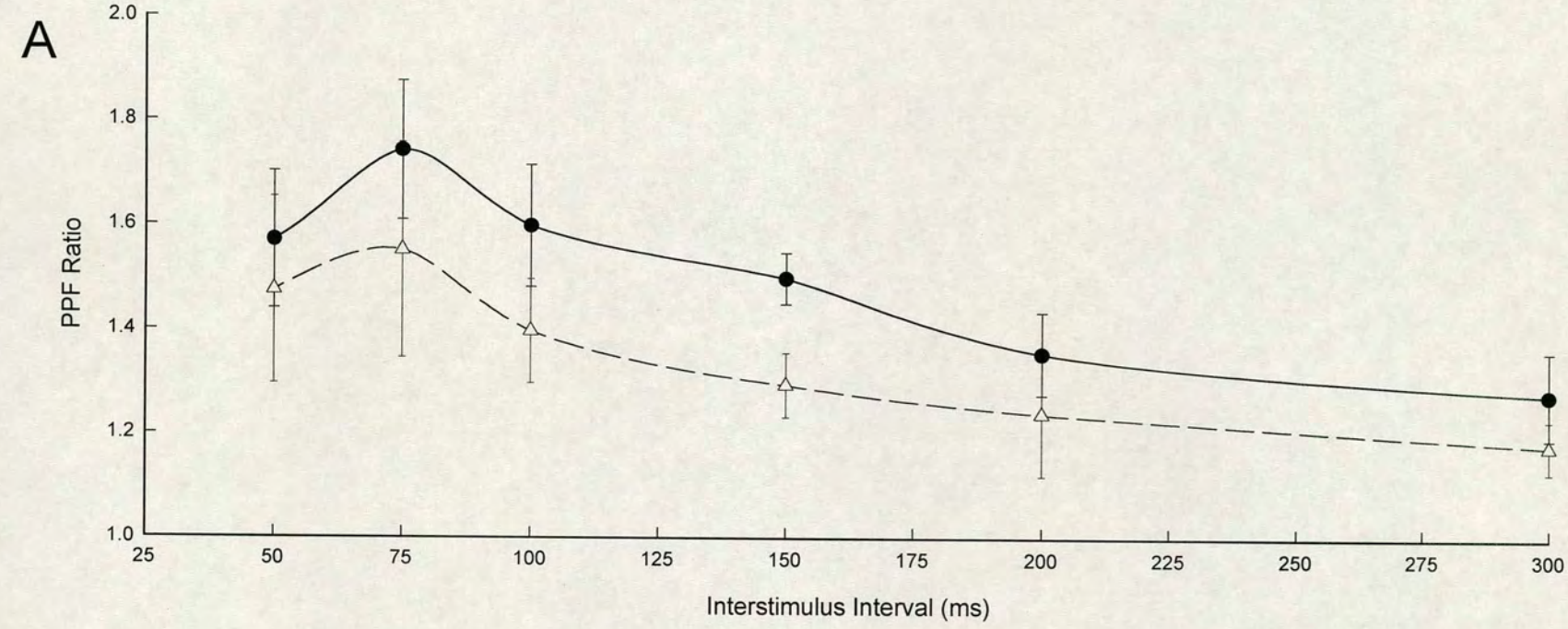
A The averaged profile of PPF at inter-stimulus intervals of 25-300ms in wild type 129/Sv (black circles) and $fyn^{129/Sv-/-}$ (white triangles) animals.

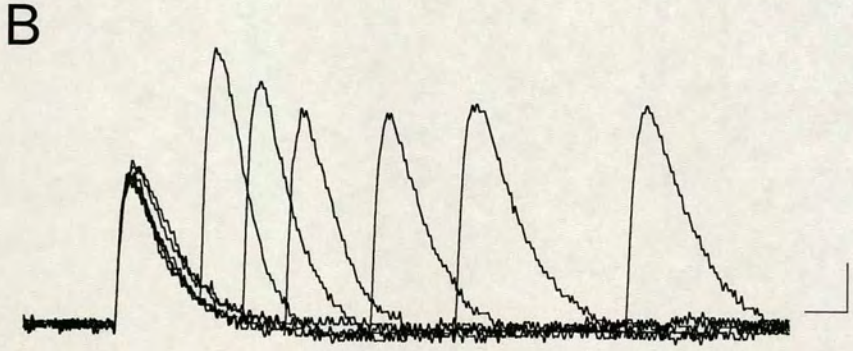
B Examples of intracellular EPSPs in $fyn^{129/Sv-/-}$ animals.

C Examples of intracellular EPSPs in wild type 129/Sv animals.

Error bars indicate the mean ± standard error.

Scale bars, 1mV, 25ms,


A
PPF Ratio
2.0
1.8
1.6
1.4
1.2
1.0
25
50
75
100
125
150
175
200
225
250
275
300
Interstimulus Interval (ms)


B

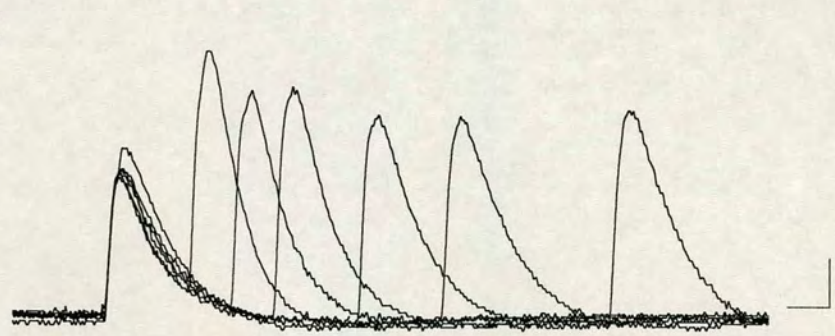

C

Table 14 - PPF ratios in AMPA receptor mediated intracellular recordings

Interval (ms)	*Wild Type PPF Ratio*	*Wild Type S.E.M.*	*Fyn PPF Ratio*	*Fyn S.E.M.*
50	1.571	0.131	1.475	0.178
75	1.741	0.132	1.551	0.205
100	1.598	0.116	1.396	0.099
150	1.498	0.049	1.293	0.062
200	1.355	0.079	1.238	0.119
300	1.275	0.083	1.177	0.050

Figure 24 **The effects of extracellular calcium manipulations of 129/Sv animals (See 5.6.).**

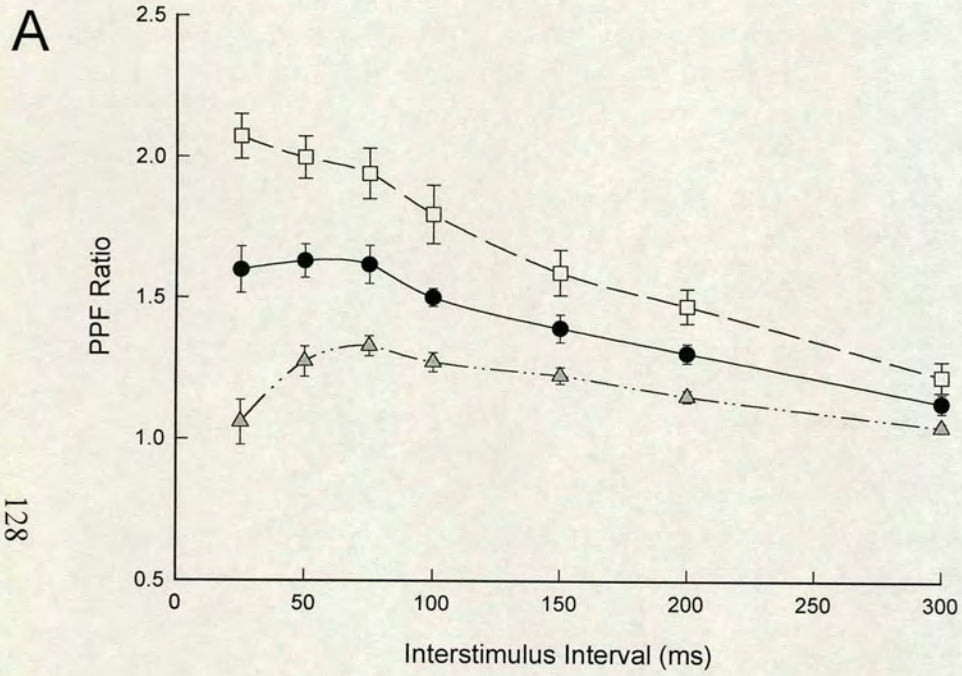
A The effects of high (gray triangles) and low (white squares) calcium containing solutions on the profile of PPF in wild type 129/Sv animals. The control response is shown by the black circles.

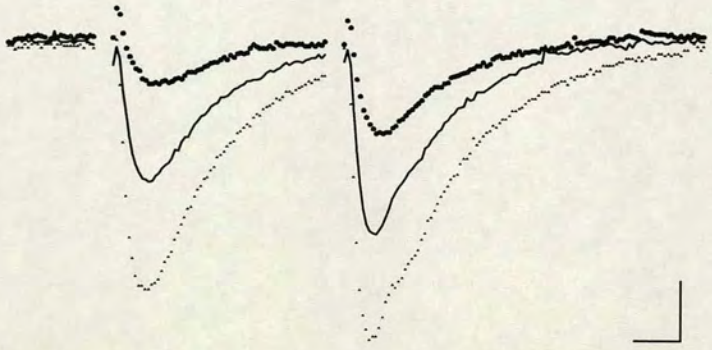
B Example traces of fEPSPs in wild type 129/Sv animals upon the application of high (triangles) and low (circles) calcium solutions. Scale bar, 0.25mV, 5ms.

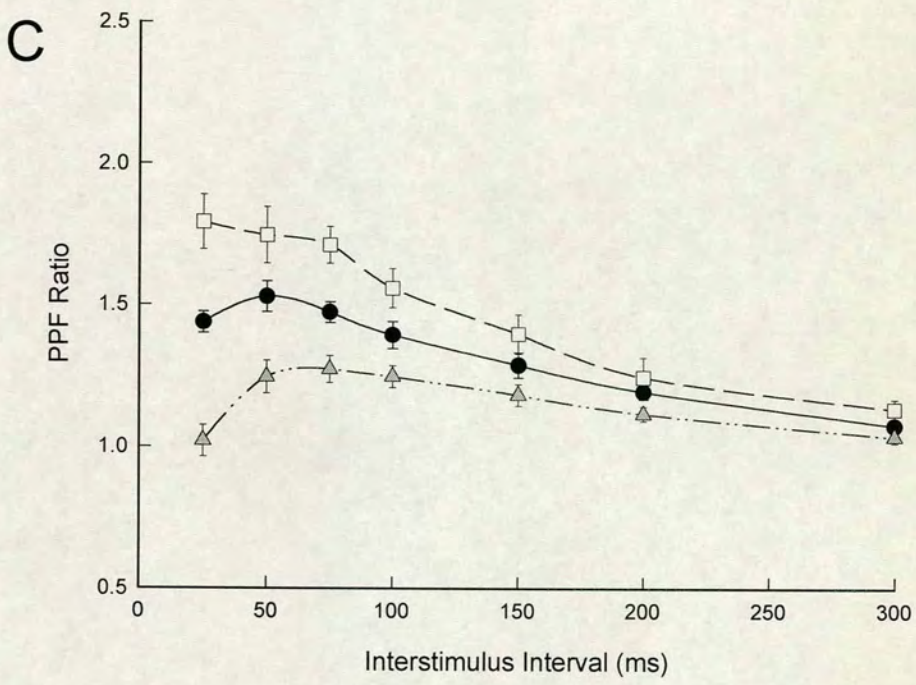
C The effects of high (gray triangles) and low (white squares) calcium containing solutions on the profile of PPF in $fyn^{129/Sv-/-}$ animals. The control response is shown by the black circles.

D Example traces of fEPSPs in $fyn^{129/Sv-/-}$ animals upon the application of high (triangles) and low (circles) calcium solutions. Scale bar, 0.25mV, 5ms.

Error bars indicate the mean ± standard error.


A
PPF Ratio
2.5
2.0
1.5
1.0
0.5
0
50
100
150
200
250
300
Interstimulus Interval (ms)


B


C
PPF Ratio
2.5
2.0
1.5
1.0
0.5
0
50
100
150
200
250
300
Interstimulus Interval (ms)

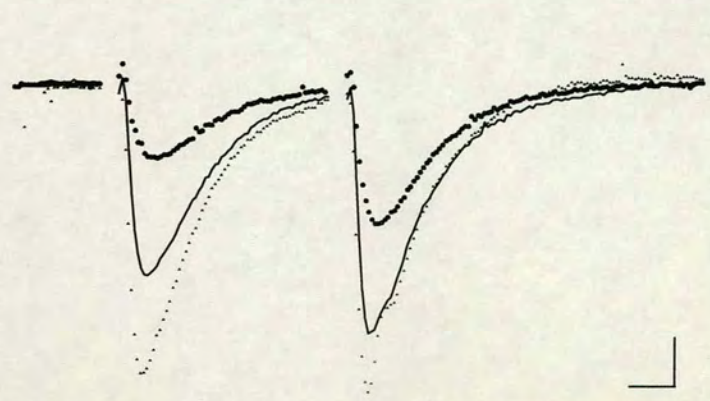

D

Table 15 – PPF ratios in normal and low calcium solutions in wild type 129/Sv animals

Interval (ms)	*Wild Type Ratio Normal Calcium*	*Wild Type S.E.M. Normal Calcium*	*Wild Type Ratio Low Calcium*	*Wild Type S.E.M. Low Calcium*
25	1.600	0.082	2.072	0.079
50	1.630	0.059	1.997	0.074
75	1.617	0.067	1.940	0.088
100	1.501	0.031	1.796	0.104
150	1.392	0.049	1.589	0.080
200	1.305	0.033	1.472	0.061
300	1.131	0.032	1.224	0.054

Table 16 – PPF ratios in normal and high calcium solutions in wild type 129/Sv animals

Interval (ms)	*Wild Type Ratio Normal Calcium*	*Wild Type S.E.M. Normal Calcium*	*Wild Type Ratio High Calcium*	*Wild Type S.E.M. High Calcium*
25	1.600	0.082	1.059	0.079
50	1.630	0.059	1.274	0.053
75	1.617	0.067	1.329	0.035
100	1.501	0.031	1.272	0.033
150	1.392	0.049	1.225	0.029
200	1.305	0.033	1.153	0.020
300	1.131	0.032	1.047	0.014

Table 17 – PPF ratios in normal and low calcium solutions in $fyn^{129/Sv-/-}$ animals

Interval (ms)	*Fyn Ratio Normal Calcium*	*Fyn S.E.M. Normal Calcium*	*Fyn Ratio Low Calcium*	*Fyn S.E.M. Low Calcium*
25	1.439	0.037	1.793	0.096
50	1.529	0.054	1.746	0.099
75	1.474	0.036	1.711	0.064
100	1.394	0.047	1.558	0.068
150	1.289	0.044	1.398	0.069
200	1.196	0.025	1.247	0.069
300	1.078	0.020	1.136	0.033

Table 18 – PPF ratios in normal and high calcium solutions in $fyn^{129/Sv-/-}$ animals

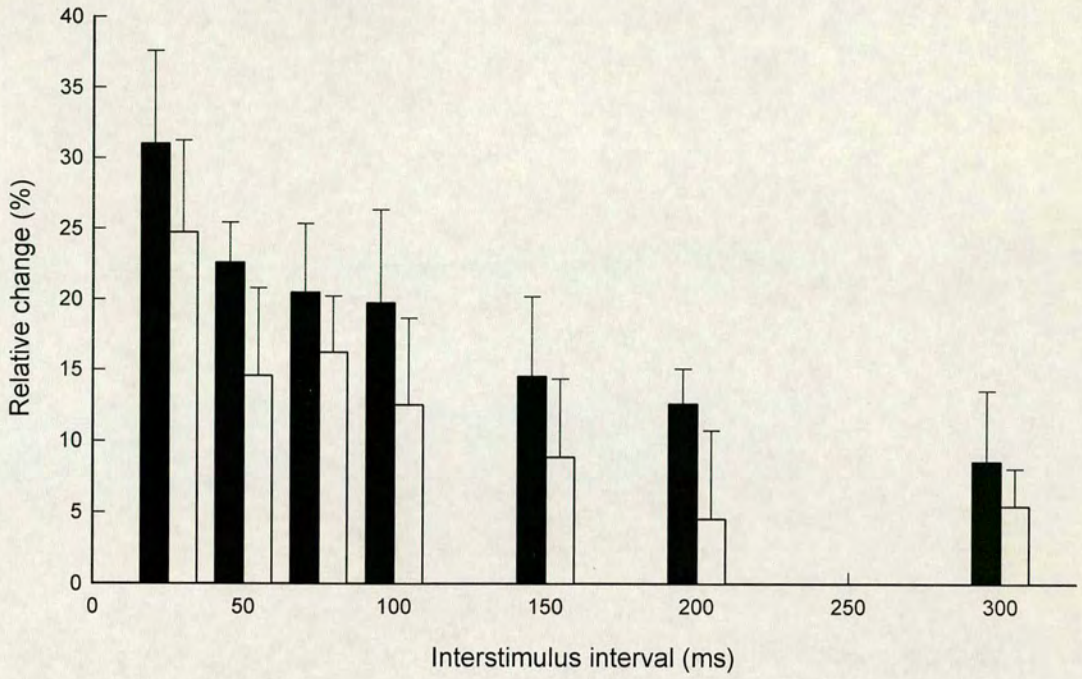
Interval (ms)	*Fyn Ratio Normal Calcium*	*Fyn S.E.M. Normal Calcium*	*Fyn Ratio High Calcium*	*Fyn S.E.M. High Calcium*
25	1.439	0.037	1.021	0.055
50	1.529	0.054	1.246	0.058
75	1.474	0.036	1.273	0.047
100	1.394	0.047	1.247	0.038
150	1.289	0.044	1.184	0.037
200	1.196	0.025	1.117	0.025
300	1.078	0.020	1.038	0.020

Figure 25 **Graphs of the relative changes seen in the calcium manipulations (See 5.6.).**

A The effects of high calcium solution on the relative change of PPF ratio for both wild type (black bars) and $fyn^{129/Sv-/-}$ (white bars). Error bars indicate the mean ± standard error.

B The effects of low calcium solution on the relative change of PPF ratio for both wild type (black bars) and $fyn^{129/Sv-/-}$ (white bars). Error bars indicate the mean ± standard error.

A


Relative change (%)
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
300
Interstimulus interval (ms)

B

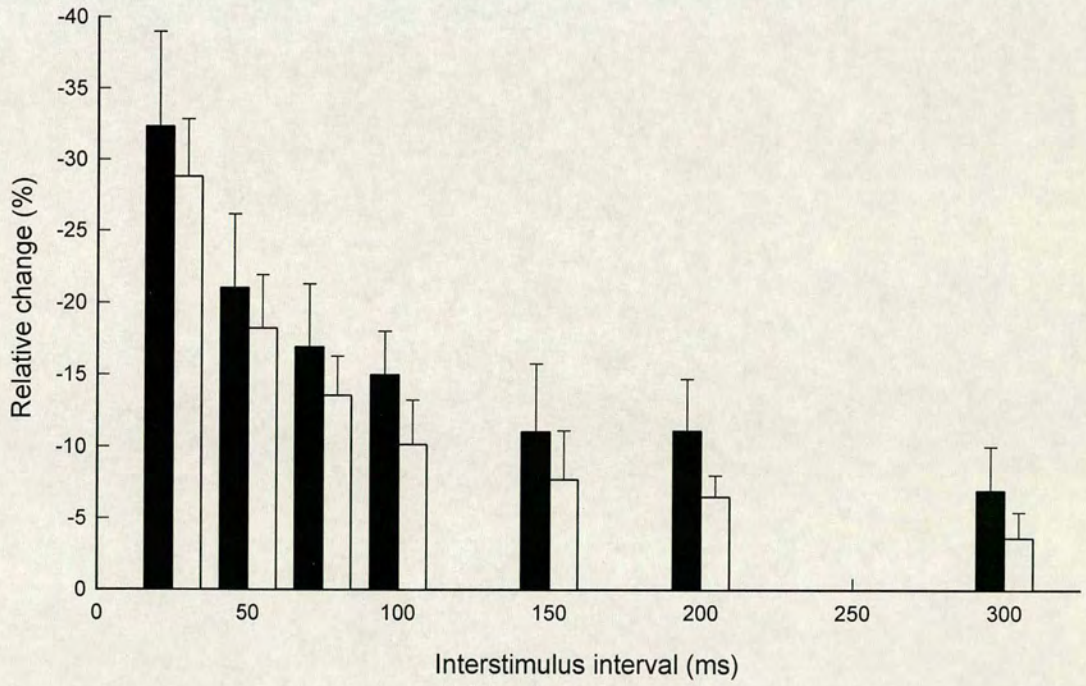

Relative change (%)
-40
-35
-30
-25
-20
-15
-10
-5
0
0
50
100
150
200
250
300
Interstimulus interval (ms)

Table 19 – Relative percentage change in high calcium solution in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

Interval (ms)	*Wild Type Relative Change (%)*	*Wild Type S.E.M.*	*Fyn Relative Change (%)*	*Fyn S.E.M.*
25	31.018	6.582	24.755	6.487
50	22.613	2.840	14.577	6.208
75	20.460	4.902	16.236	3.977
100	19.716	6.618	12.521	6.123
150	14.543	5.643	8.895	5.466
200	12.609	2.477	4.563	6.168
300	8.568	4.955	5.452	2.611

Table 20 – Relative percentage change in low calcium solution in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

Interval (ms)	*Wild Type Relative Change (%)*	*Wild Type S.E.M.*	*Fyn Relative Change (%)*	*Fyn S.E.M.*
25	-32.323	6.648	-28.788	4.035
50	-21.004	5.140	-18.190	3.713
75	-16.896	4.381	-13.548	2.708
100	-14.972	3.030	-10.147	3.085
150	-11.040	4.698	-7.703	3.405
200	-11.114	3.584	-6.511	1.471
300	-6.942	3.094	-3.634	1.805

5.7. Modulation of presynaptic release by carbachol

The effects of a mAChR agonist carbachol on PPF at an ISI of 50ms were studied in hippocampal slices from wild type 129/Sv and fyn$^{129/Sv-/-}$ mice. Hippocampal slices were exposed to fifteen minute bath applications of carbachol at varying concentrations (0.03, 0.1, 0.3, 1, 3, and 10 μM). Both wild type 129/Sv (n = 8) and fyn$^{129/Sv-/-}$ (n = 5) slices showed an increased PPF ratio at carbachol concentrations above 0.3 μM. A difference existed between the PPF ratios seen in fyn$^{129/Sv-/-}$ and wild type 129/Sv mice at all carbachol concentrations (see Figure 26A, Table 21). Low concentrations of carbachol (0.03 & 0.3 μM) increased the slope of the fEPSP in fyn$^{129/Sv-/-}$ mice by 18 ± 7 % and 18 ± 12 % respectively (Figure 26B). Higher concentrations (1-10 μM) caused a reduction in the slope of the fEPSP (between 33 and 56 %, see Table 22). In contrast, low concentrations of carbachol had no effect on wild type 129/Sv slices, however a reduction in fEPSP slope between 32 and 48 % was seen at higher concentrations (Figure 26B and Table 22). A slight difference in fEPSP slope was seen between wild type 129/Sv and fyn$^{129/Sv-/-}$ slices at carbachol concentrations of 0.03 μM and 0.1 μM (see Table 22). These effects were completely reversible upon a fifteen minute bath application of the mAChR antagonist, atropine (2μM) which also produced an enhancement of the fEPSP size above the baseline response (data not shown). The effect of genotype was also apparent in this experiment, tested using a one way ANOVA ($P < 0.001$, $F = 6.879$).

5.8. Modulation of presynaptic release by adenosine

A non-hydrolysable analogue of adenosine (2-chloroadenosine, CADO 0.25 μM) was bath applied for fifteen minutes to slices from wild type 129/Sv and fyn$^{129/Sv-/-}$ animals. This was used in preference to adenosine as it not broken down whilst exposed to the slice and thus produces more reliable results. Paired pulse facilitation was measured at the 50 ms ISI. CADO produced a reduction of 37 ± 5 % in the fEPSP slope in wild type 129/Sv slices and 32 ± 3 % in fyn$^{129/Sv-/-}$ slices (see Figure 27B and Table 24). The associated change in PPF ratio showed no difference

between $fyn^{129/Sv-/-}$ (n = 10) and wild type 129/Sv slices (n = 10, Figure 27A, Table 23). Reversal of these effects by a fifteen minute bath application of a specific A_1 adenosine receptor antagonist (DPCPX, 200 nM) increased the fEPSP slope above control levels by 41 ± 11 % in wild type 129/Sv slices and 33 ± 12 % in $fyn^{129/Sv-/-}$ slices (see Figure 27B and Table 24). No difference in the ratio of PPF in DPCPX was found between wild type 129/Sv and $fyn^{129/Sv-/-}$ slices (see Table 23). A one way ANOVA test confirmed the effect of genotype under each condition in this experiment ($P < 0.001$, $F = 10.288$).

Figure 26 **The effects of carbachol on PPF at the 50ms inter-stimulus interval in 129/Sv animals (See 5.7.).**

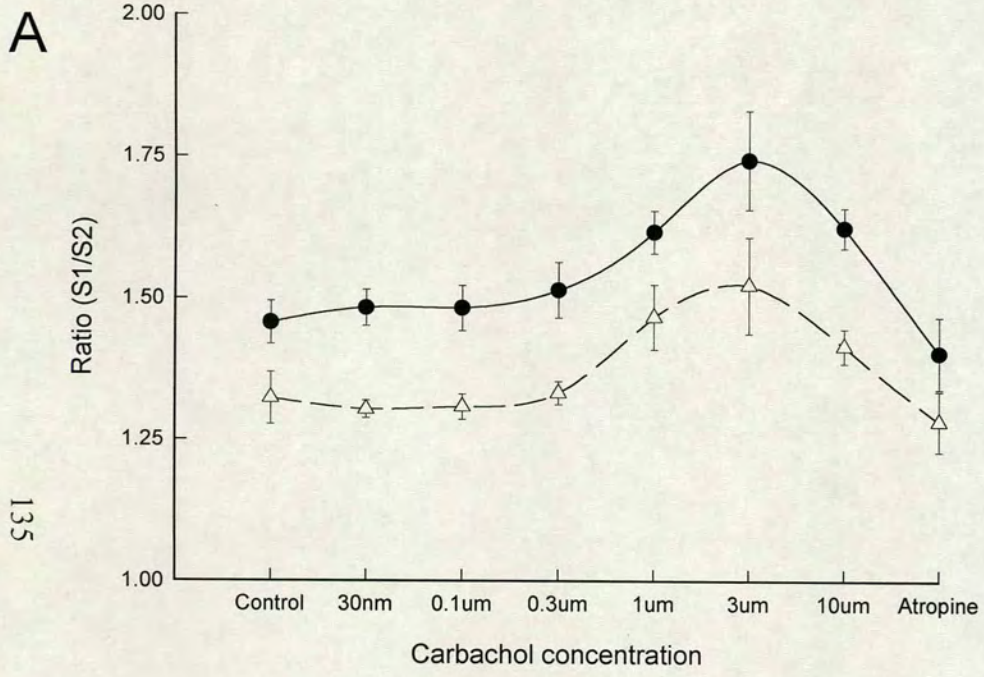
A The ratio of PPF was modulated by the application of carbachol in both the wild type 129/Sv (black circles) and $fyn^{129/Sv-/-}$ (white triangles), which was reversed by atropine.

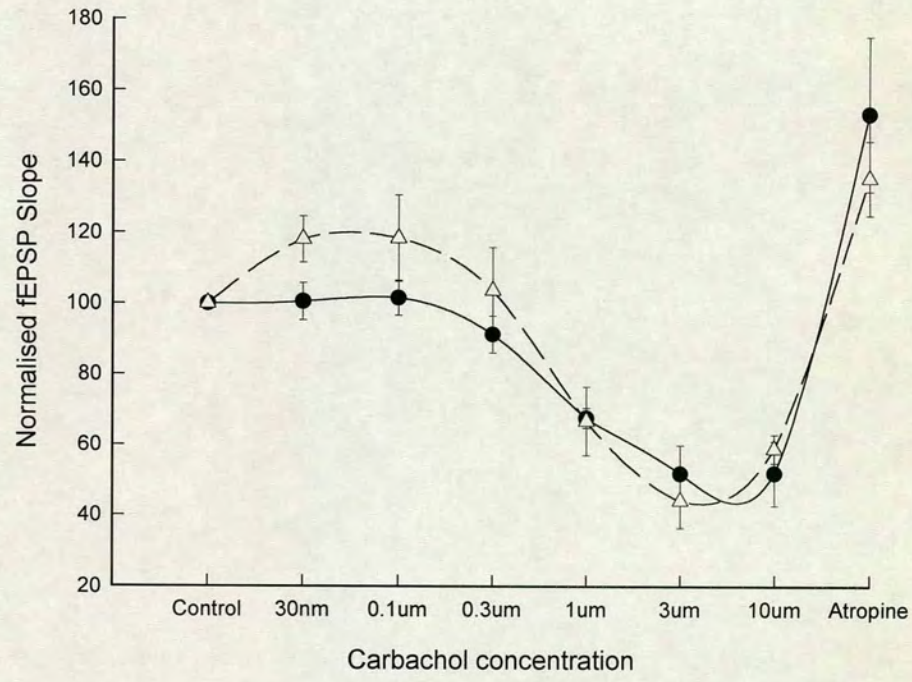
B The percentage change from normalised fEPSP slope is plotted against carbachol concentration for both wild type 129/Sv (black circles) and $fyn^{129/Sv-/-}$ animals (white triangles).

C Example traces of the effects of carbachol (circles) and atropine (triangles) on wild type 129/Sv fEPSPs. Scale bar 0.25, 5ms.

D Example traces of the effects of carbachol (circles) and atropine (triangles) on $fyn^{129/Sv-/-}$ fEPSPs. Scale bar 0.25, 5ms.

Error bars indicate the mean ± standard error.


A
2.00
1.75
1.50
1.25
1.00
Ratio (S1/S2)
Control
30nm
0.1um
0.3um
1um
3um
10um
Atropine
Carbachol concentration


B
180
160
140
120
100
80
60
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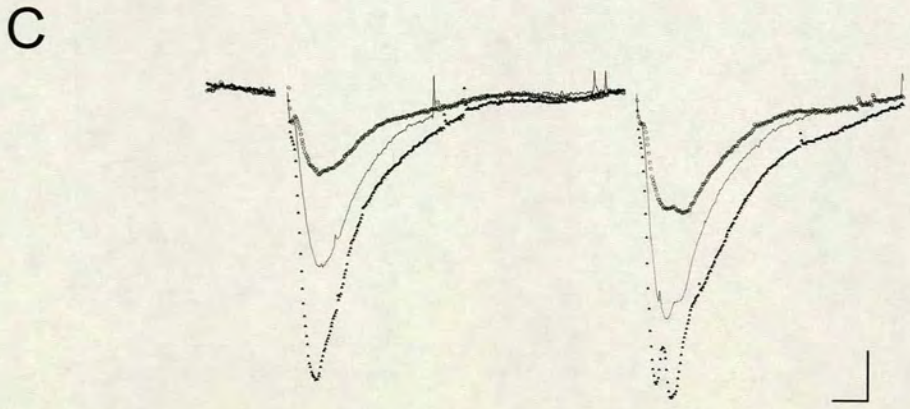
20
Normalised fEPSP Slope
Control
30nm
0.1um
0.3um
1um
3um
10um
Atropine
Carbachol concentration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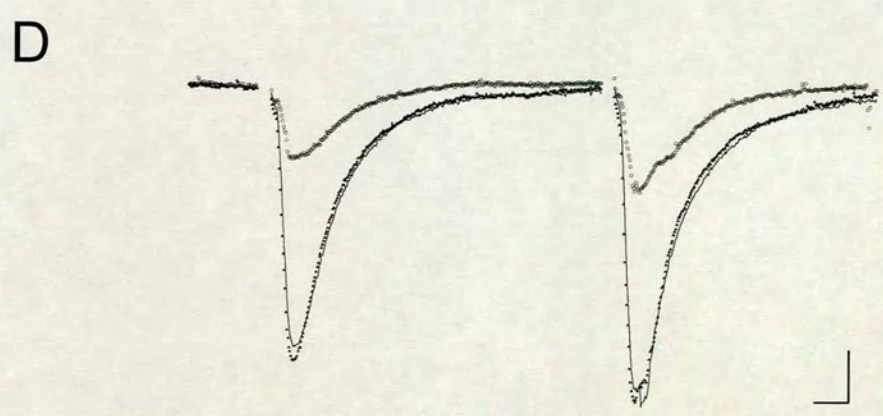

D

Table 21 – PPF ratios in carbachol experiments in fyn$^{129/Sv-/-}$ and wild type 129/Sv animals

Carbachol Concentration (μM)	*Wild Type PPF Ratio*	*Wild Type S.E.M.*	*Fyn PPF Ratio*	*Fyn S.E.M.*
Control	1.457	0.038	1.323	0.046
0.03	1.483	0.031	1.303	0.015
0.10	1.481	0.039	1.308	0.022
0.30	1.514	0.049	1.332	0.020
1.00	1.617	0.037	1.467	0.057
3.00	1.743	0.086	1.523	0.085
10.0	1.624	0.035	1.415	0.030
Atropine	1.403	0.063	1.282	0.053

Table 22 – Normalised EPSP slopes in carbachol experiments in fyn$^{129/Sv-/-}$ and wild type 129/Sv animals

Carbachol Concentration (μM)	*Wild Type Normalised EPSP Slope (%)*	*Wild Type S.E.M.*	*Fyn Normalised EPSP Slope (%)*	*Fyn S.E.M.*
Control	100.000	0.000	100.000	0.000
0.03	100.650	5.212	118.177	6.495
0.10	101.663	4.952	118.500	12.105
0.30	91.3485	5.261	103.747	12.145
1.00	67.6252	2.784	66.8016	9.781
3.00	51.7709	8.161	44.0971	7.563
10.0	51.8982	9.234	58.9108	4.079
Atropine	153.357	21.692	135.375	10.461

Figure 27 **The effects of CADO on the PPF at the 50ms inter-stimulus interval in 129/Sv animals (See 5.8.)**

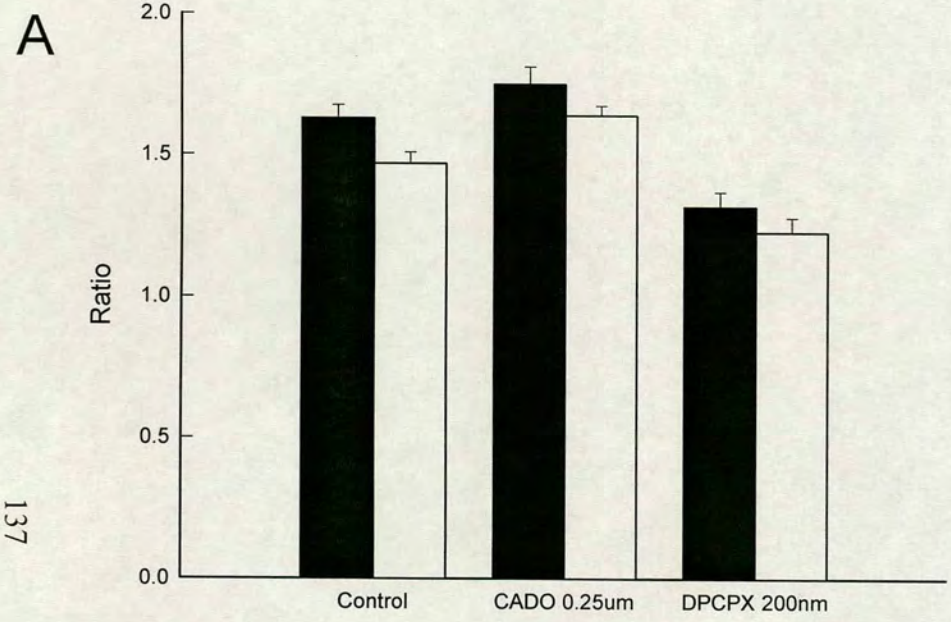
A The application of CADO (0.25uM) and DPCPX (200nM) modulated the PPF ratio in both wild type129/Sv (black bars) and $fyn^{129/Sv-/-}$ (white bars) to the same extent.

B The same data as presented in A, normalised to the control response.

C Example traces of the effects of CADO (circles) and DPCPX (triangles) on wild type 129/Sv fEPSPs. Scale bar 0.25, 5ms.

D Example traces of the effects of CADO (circles) and DPCPX (triangles) on $fyn^{129/Sv-/-}$ fEPSPs. Scale bar 0.25, 5ms.

Error bars indicate the mean ± standard error.


A
Ratio
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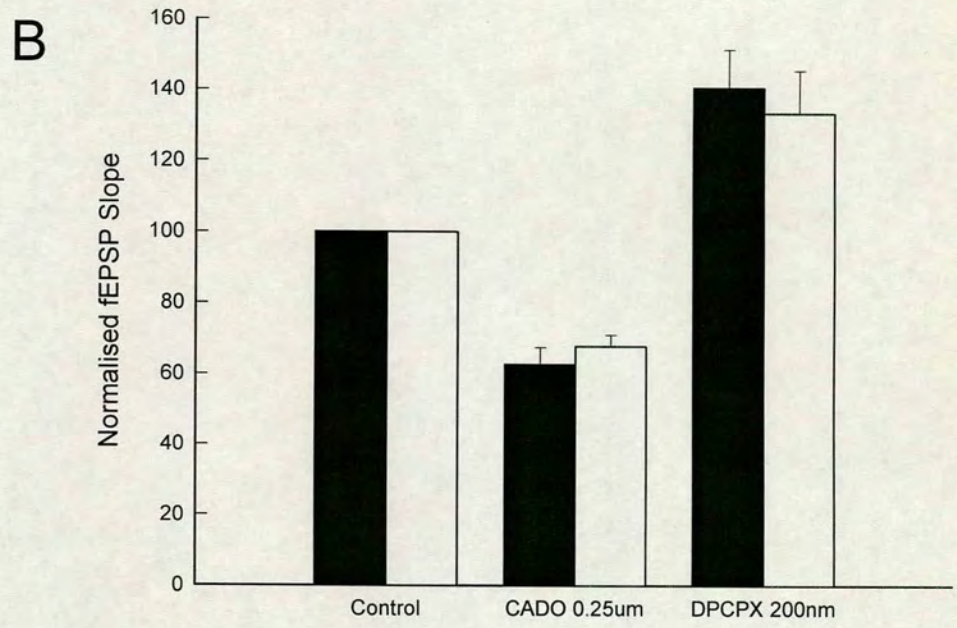
1.5
1.0
0.5
0.0
Control
CADO 0.25um
DPCPX 200nm


B
Normalised fEPSP Slope
160
140
120
100
80
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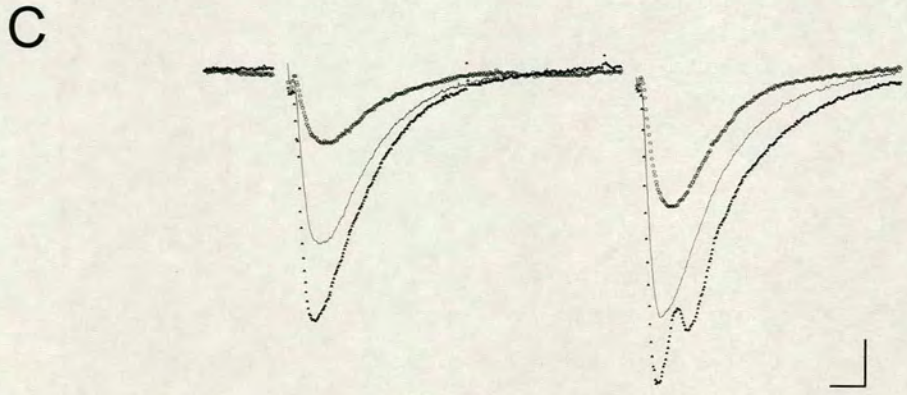
40
20
0
Control
CADO 0.25um
DPCPX 200nm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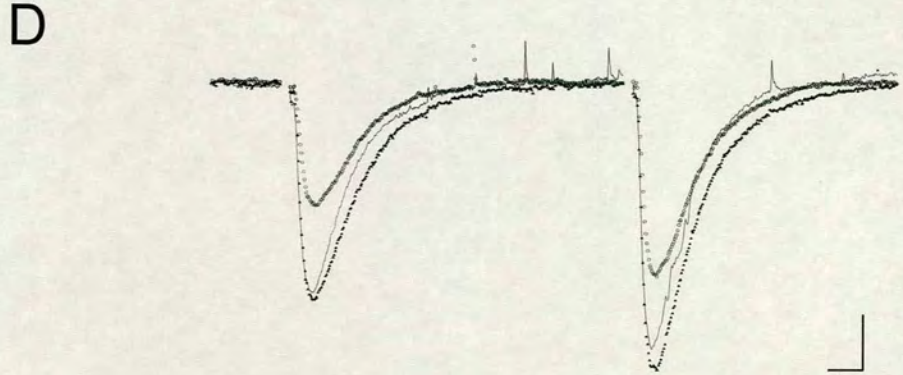
D

Table 23 – PPF ratios in CADO experiments in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

	Wild Type PPF Ratio	*Wild Type S.E.M.*	*Fyn PPF Ratio*	*Fyn S.E.M.*
Control	1.627	0.046	1.467	0.041
CADO	1.747	0.062	1.636	0.034
DPCPX	1.316	0.052	1.226	0.051

Table 24 – Normalised EPSP slopes in CADO experiments in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals

	Wild Type Normalised EPSP Slope (%)	*Wild Type S.E.M.*	*Fyn Normalised EPSP Slope (%)*	*Fyn S.E.M.*
Control	100.000	0.000	100.000	0.000
CADO	62.695	4.800	67.722	3.265
DPCPX	140.505	10.796	133.361	12.090

5.9. Biochemical analysis of presynaptic proteins in fyn$^{129/Sv-/-}$ mutants.

Materials

The resin used in this study was Protein G-Sepharose from Pharmacia (U.K.). Antibodies used for Western blotting were the mouse monoclonals: anti-synaptophysin from Roche Molecular Biochemicals (U.K.), anti-synaptogyrin and peroxidase-linked anti-phosphotyrosine RC20 from Transduction Laboratories (U.S.A.).

Protein extraction

Whole mouse forebrains from wild type 129/Sv and fyn$^{129/Sv-/-}$ mice were homogenised on ice in an SDS containing buffer (SDS buffer) composed of 50 mM Tris pH 9.0, 1 mM sodium ortho-vanadate, 20 μM zinc chloride, 4% sodium dodecyl sulfate, 0.5 mM PMSF, 2 μg/ml Aprotinin and 2 μg/ml Leupeptin at 0.38 g wet weight per 7 ml cold buffer. The extracts were boiled for 30 minutes and clarified by centrifugation at 13.000xg for 30 min at room temperature and used for immunoprecipitation experiments.

Immunoprecipitation

Small-scale experiments were performed by mixing 50 μl extract with 950 μl of NP40 buffer (Tris buffered saline, 1% Nonidet P40, 1mM sodium ortho-vanadate) and adding antibody as outlined in the Figure 28. 15 μl Protein G-Sepharose was added after an incubation of 4 hours at 4°C followed by a further incubation overnight at 4°C with constant agitation. The resins were then washed with four cycles of 0.5 ml NP40 buffer each and subjected to SDS-PAGE analysis.

Western blotting

Protein samples were subjected to reducing SDS-PAGE and transferred to PVDF membrane (BioRad) at 4°C for 90 minutes at 75 V in 10% (v/v) Methanol, 10 mM CAPS pH 11.0. Dilution of primary antibodies was between 1:100 and 1:1000 depending on the quality of the immunoglobulins (IgGs). Detection of signals was done using peroxidase-linked secondary IgGs or the peroxidase-protein directly

linked to the IgG and enhanced chemiluminescence. Re-probing of once used western blots was done by incubating the membrane in 200 mM glycine pH2.5, 0.05% Tween 20 for 90 minutes at 80°C followed by transfer of the blots into phosphate buffered saline (PBS), followed by several washing steps in PBS. The western blots were then used for detection of antigens using the protocol outlined above.

Results

To determine if fyn is responsible for the tyrosine phosphorylation of presynaptic vesicle proteins the state of phosphorylation was analysed as shown in Figure 28. Both presynaptic vesicle proteins, synaptogyrin and synaptophysin, showed no difference on the level of tyrosine phosphorylation in $fyn^{129/Sv-/-}$ mice compared to wild type 129/Sv controls. This can be seen by the bands at 33 kDa (synaptophysin, lanes 2 and 3, Figure 28) and 29 kDa (synaptogyrin, lanes 4 and 5, Figure 28). Controls were as expected (lane 6 for a negative control and lanes 1 and 7 for positive controls). Both proteins did show tyrosine phosphorylation but no difference could be seen between the wild type 129/Sv and $fyn^{129/Sv-/-}$ animals. Equal sample sizes of protein extracts were used in all cases. The presence of synaptogyrin was established by re-probing the western blot with anti-synaptogyrin, however the anti-synaptophysin antibody was apparently unable to recognise the native form of the protein in this assay (data not shown). This indicates that this anti-body is unsuitable for western blotting. However, since positive signals for both molecules were seen in the anti-tyrosine-phosphorylation blots, it can be concluded that there is no difference in the phosphorylation state of both molecules regardless of the fyn mutation.

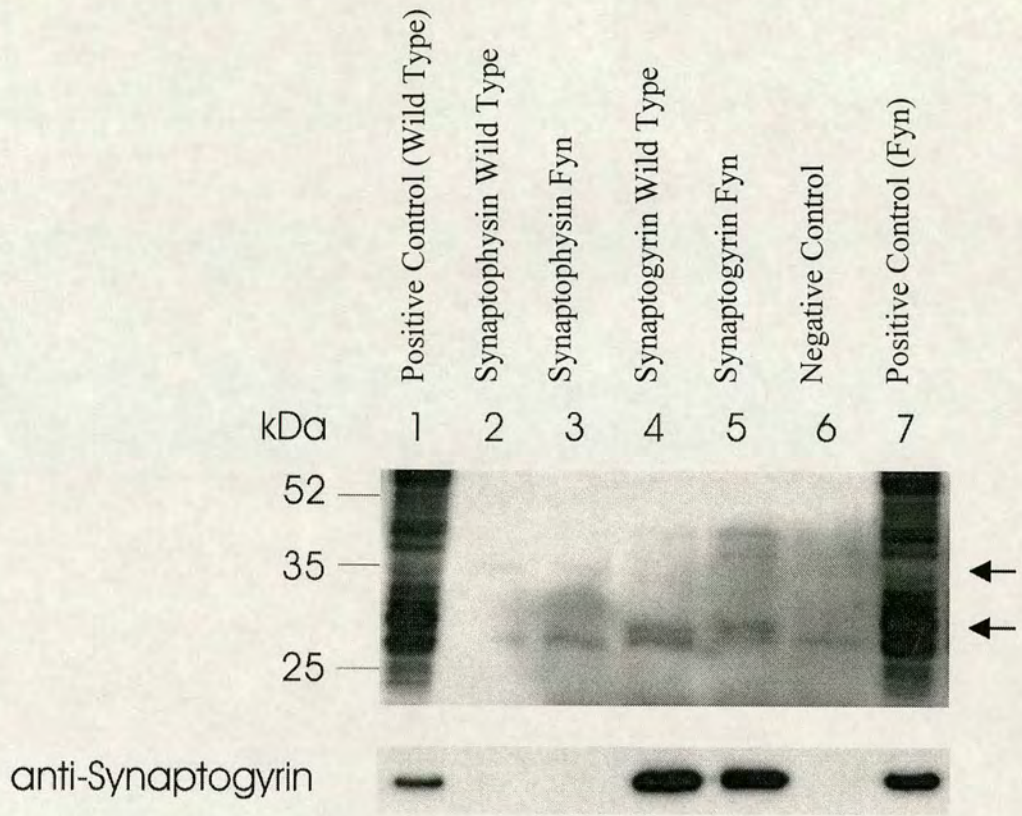

Figure 28 - Phosphorylation states of presynaptic proteins in $fyn^{129/Sv-/-}$ and wild type 129/Sv animals.

Legend - Mouse brain extracts were analysed for the tyrosine phosphorylation state of both synaptogyrin and synaptophysin by immunoprecipitation using 5 µl anti-synaptophysin and 1ml wild type 129/Sv (lane 2) and $fyn^{129/Sv-/-}$ brain extracts (lane 3) or 1 µl anti-synaptogyrin and the same amounts of extracts (lane 4 wild type 129/Sv, lane 5 $fyn^{129Sv-/-}$). Controls were incorporated by using ProteinG-sepharose alone without IgG and wild type129/Sv extract (lane 6). Positive controls were either untreated wild type 129/Sv (lane 1) or fyn129/Sv-/- brain extracts (lane 7). Molecular weight markers are included on the left of the blot developed against anti-phosphotyrosine (upper panel). The presence of synaptogyrin was determined by stripping and re-probing the blot for the molecule.

Chapter VI

Discussion

Short-term plasticity

6.1. Introduction

Paired pulse facilitation (PPF) is an established form of short-term plasticity seen in pre-synaptic terminals throughout the peripheral and central nervous system, including excitatory synapses in the hippocampus (Wu and Saggau, 1994, Zucker, 1989). Two stimuli delivered in close temporal succession causes a facilitation of the second response. This facilitation of presynaptic transmitter release following paired stimuli is explained as follows: the first action potential invades the presynaptic terminal, activating voltage gated calcium channels (in the active zone where the vesicles docking and release sites exist) which allow a large, transient calcium ion influx that initiates transmitter release and produces a postsynaptic response. Initially a high concentration of calcium is present around the active zone, which then diffuses towards the interior of the terminal no longer affecting transmitter release. The calcium is then taken up into organelles and excreted from the presynaptic terminal by surface membrane pumps. The diffusion, sequestering and extrusion of calcium is a process which occurs in the order of seconds, whilst action potential invasion of the presynaptic terminal can occur in the order of milliseconds.

This means that a second action potential can invade the presynaptic terminal before the intracellular calcium levels are returned to normal resting levels. In this case the residual calcium concentration, which in itself is too small to initiate release, summates with the calcium influx from the second action potential and increases the amount of transmitter released and facilitates the second postsynaptic response. The relationship between neurotransmitter release and intracellular calcium concentration is non-linear. This process has been termed the residual calcium hypothesis proposed by Katz and Miledi in 1968 and is generally used to explain the facilitation seen with paired stimuli (del Castillo and Katz, 1954, Magleby, 1987, Zucker, 1989).

PPF is usually expressed as the ratio of the peak amplitude or slope of the second response compared to the first response. An increase in this ratio is normally associated with a decrease in the probability of transmitter release from the

presynaptic terminal (P_r); conversely a decrease in this ratio indicates an increase in P_r. In the hippocampus, the release of neurotransmitter is mediated predominately by N and Q type voltage gated calcium channels, located in the presynaptic active zone (Wheeler et al., 1994).

6.2. Extracellular paired pulse facilitation in $fyn^{-/-}$ and $fyn^{129/Sv-/-}$ mice

In the present study mice lacking fyn kinase, from both the hybrid and inbred genetic backgrounds, displayed a significant decrease in the PPF ratio that was seen at most inter-stimulus intervals between 25 – 300ms. On the basis of classical interpretations of PPF (the residual calcium hypothesis of Katz and Miledi), this may indicate that these mice have a higher initial release probability than wild type animals. This results is in contrast to the LTP results, in which a strain variation was seen. Therefore, it appears that the mechanisms by which fyn mediates its effects on LTP are different to those mediating PPF.

This effect was not seen in the Grant et al. 1992 study. PPF of the fEPSP was only measured at one interval of 50ms and no statistical difference was seen in the level of PPF between any of the src family mutants (fyn, yes, src, and abl) compared to wild type mice (no figures quoted). It is interesting to note however, that there was variation in the level of facilitation seen between the groups tested, with $fyn^{-/-}$ mice displaying both the lowest level of facilitation and the largest standard error in the animals tested (Grant et al., 1992).

To elucidate the possible mechanisms, by which fyn mediates this apparent presynaptic change, the profile of paired pulse facilitation was examined under various experimental conditions. These experiments are discussed below.

6.3. Age dependent effects on paired pulse facilitation in fyn$^{129/Sv-/-}$ mice

Synaptic transmission and the level of facilitation seen in PPF are developmentally regulated. Cell proliferation is nearly complete at birth in area CA1 of the hippocampus (Bayer, 1980) whilst most synaptic contacts form up until the fourth postnatal week (Baudry et al., 1981). In neonates (4-8 days old) PPF is absent and associated with a probability of release which is close to unity, thus the amount of transmitter released on the first and second pulses will be equivalent, as the probability of release cannot be enhanced. The probability of release progressively decreases over the first weeks of development and PPF becomes apparent in animals between 2 and 3 weeks old (Bolshakov and Seigelbaum, 1995). There is no apparent change in axon excitability or extracellular fibre volley area in animals from post natal days (P) 15-21 compared to animals from P29-35, however a significant increase in the EPSP slope is seen between these two groups (Dumas and Foster, 1995). The associated changes in PPF and EPSP size during the first few weeks postnatally is presumably due to the formation and consolidation or elimination of synaptic contacts as activity dependent changes occur.

There are known effects of age on the induction of LTP in fyn mutants. Animals under the age of 14 weeks of age show normal LTP and the reported impairment in LTP induction is only detectable after 14 weeks of age. This effect has been explained by the compensation of fyn tyrosine kinase function by up-regulated expression of src tyrosine kinase in animals less than 14 weeks of age. Over 14 weeks of age however, levels of src expression fall dramatically, and the underlying impairment of LTP induction is uncovered (Kojima et al., 1997, Grant et al., 1995). In this study, a developmental change was seen in the PPF ratio in both wild type 129/Sv and fyn$^{129/Sv-/-}$ animals. In both cases, the PPF ratio was greater in young (< 6 weeks of age) animals as compared to older animals (> 14 weeks of age). A significant difference between the level of PPF in wild type 129/Sv and fyn$^{129/Sv-/-}$ animals was still apparent at this younger age however, indicating that the loss of fyn has effects on PPF, which are independent of the effects on LTP (adding weight to the proposal that fyn mediates LTP and PPF via different mechanisms). Src up-

regulation in fyn knockouts seems to be able to compensate for the reduction in LTP at young ages but the reduction in PPF is apparent at times when src expression is both high (in younger animals) and low (in older animals). Thus src up-regulation cannot account for the decreased PPF ratio seen in $fyn^{129/Sv-/-}$ animals, but may contribute to the increase in PPF seen at the younger age in both groups of animals by some an unknown mechanism which decreases the probability of transmitter release.

6.4. Intracellular paired pulse facilitation in $fyn^{129/Sv-/-}$ mice

Intracellular recordings of AMPAR mediated EPSPs in CA1 pyramidal cells showed a general trend for $fyn^{129/Sv-/-}$ cells to show a decreased PPF ratio compared to wild type 129/Sv cells, although statistical significance was only achieved at one ISI of 150ms. The recordings of intracellular PPF are complicated by trial to trial variation that exists in responses to successive stimuli. Both PPF and PPD can be seen in individual cells between trials. The averaged response in all cases showed PPF, as is the case extracellularly. This effect has been seen in other reports of intracellular PPF recordings, and is explained by the initial probability of release of individual synapses. If the initial release probability for the first pulse is high, PPD is seen, however if the initial release probability is low, PPF is seen. The percentage of trials displaying PPD is increased if the probability of release is raised and smaller when the probability of release is lowered (Debanne et al., 1996). This variation in facilitation or depression may mask any changes in the fyn mutants. The results presented in this thesis were generated from the averages of 10 successive trials at each individual inter-stimulus interval. The averaging of PPD and PPF in the same data sets will always tend the data towards a mean level that may explain the lack of significant effect in the intracellular data. However, if fyn mutants tend to display a higher proportion of trials exhibiting PPD instead of PPF (as would be predicted if the P_r is increased) then this would tend to reduce the mean ratio and increase significance. The analysis of the number of trials exhibiting PPF and PPD in fyn knockouts may uncover a more subtle trend towards PPD (and thus further evidence

for an increase in P_r). This may also be subject to the complications in the analysis of paired pulse data outlined below.

The small n numbers may also contribute to the lack of statistical significance. Extracellular PPF was significant, but in this case both a large numbers of slices, and an even larger population of cells was sampled, provide a better data set for statistical analysis. Another consideration is that the extracellular recordings were made in the presence of intact inhibition and NMDA receptor mediated potentials, whereas the intracellular data was purely mediated by the AMPA component. If the effects of fyn on PPF are independent of AMPA receptors, and perhaps mediated by a GABA-ergic or NMDA receptor dependent mechanism, then this effect could be explained. In this case a study of paired pulse facilitation of the NMDA and or GABA mediated components would be a future line of investigation. The use of the voltage clamp technique, which would allow control of variations in the membrane potential (that may have an effect on the level of facilitation seen), would also be a suitable line of further investigation.

6.5. Tyrosine kinase inhibitor and paired pulse facilitation in fyn$^{129/Sv-/-}$ mice

PPF in wild type 129/Sv animals was decreased upon the extracellular application of a specific src family tyrosine kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine (PP2). This inhibitor was first discovered in the search for specific inhibitors of T cell signalling pathways involving the src family kinases (Hanke et al., 1996, Traxler et al., 1997). PP2 is reversible and membrane permeable and the IC_{50} against fyn is around 5 nM in *in vitro* cell lysates. This inhibitor shows a much higher specificity than the current group of tyrosine kinase inhibitors such as genistein (IC_{50} = 25 μM), lavendustin A (IC_{50} = 500 nM) and the irreversible inhibitor herbimycin A (IC_{50} = 12μM). Many studies using inhibitors of tyrosine kinases have used genistein, which has many non-specific aspects, most notably the direct block of voltage gated sodium channels (Paillart et al., 1997) and GABAR mediated currents (Caroline Herron, Andy Boxall, Personal

communication). These non-specific effects of genistein will have dramatic effects on CNS transmission and results from electrophysiological studies using this inhibitor should be treated with caution. PP2 has not been reported to interact with any ion channel functions directly providing a more suitable inhibitor to assess the role of src family tyrosine kinases in the hippocampus. PP2 (100 nM) significantly decreased the ratio of PPF in all experiments whereas its inactive structural analogue 4-amino-7-phenylpyrazolo [3,4-*d*] pyrimidine (PP3) had no effect (Figure 21). These data provide direct evidence that PPF can be modulated by tyrosine kinases of the src family.

6.6. Manipulation of release by calcium ion concentration

The concentration of extracellular calcium significantly affects the probability of release of neurotransmitter and PPF in area CA1 of the hippocampus. The ionic driving force behind all ion flow is dependent on the relative intracellular and extracellular ion concentrations. Increasing or reducing the extracellular calcium concentration in the bathing medium can directly alter the calcium influx generated by an action potential invading the presynaptic terminal and thus modify the probability of transmitter release (Katz and Miledi, 1968, Creager et al., 1980, Mallart and Martin, 1968, Manabe et al., 1993, Zucker, 1989). In experiments presented in this thesis, raising the calcium concentration in both wild type 129/Sv and $fyn^{129/Sv-/-}$ slices caused a significant decrease in the ratio of PPF and an increase in fEPSP slope indicating that the probability of release had been increased. Conversely, lowering the calcium concentration caused a significant increase in the ratio of PPF and a decrease in fEPSP slope in both groups of animals indicating that the probability of release had been reduced (Figure 24).

However, the relative change in ratio from normal to low and high calcium containing solutions is not statistically different in wild type 129/Sv and $fyn^{129/Sv-/-}$ slices. There is a trend for $fyn^{129/Sv-/-}$ slices to show a smaller relative percentage change in ratio (and presumably transmitter release) than wild type animals at the

same calcium concentration (Figure 25). The lack of significant effect of fyn in these experiments indicates that the mechanism by which PPF is decreased in these animals is not related to the level of extracellular calcium and subsequent calcium influx to the presynaptic terminal.

A recent report details an estimated reduction of around 30% of N and T type (not L-type) calcium currents (measured with barium ion substitution) with the tyrosine kinase inhibitor genistein. However the finding that both genistein and its inactive structural analogue diadzein reduced calcium currents suggests that the effects seen may not be due to the specific inhibition of tyrosine kinase activity (see above). A smaller reduction in calcium currents was also seen with lavendustin A and herbimycin A (22% and 20% respectively) so the possibility that this modulation is tyrosine kinase specific cannot be ruled out (Morikawa et al., 1998). These effects may well be obscured in the relatively gross manipulation of changing extracellular calcium concentrations and recording averaged postsynaptic fEPSP from the hippocampal slice. It should also be noted that these inhibitors do not discriminate between src family members, thus these effects could be mediated by any member of this family of kinases.

6.7. Manipulation of release by activation of muscarinic acetylcholine receptors

Muscarinic acetylcholine receptors (mAChR) are expressed at high levels in the hippocampus, and can regulate synaptic transmission through actions on calcium, potassium and NMDAR mediated channels. mAChR activation inhibits N type VGCCs (but not P and Q type channels) and increases responses mediated by NMDARs (Cole and Nicoll, 1983, Markram and Segal, 1990, Harvey et al., 1993). In hippocampal neurones acetylcholine reduces the fEPSP (Segal, 1982, Sheridan and Sutor, 1990) by presynaptic inhibition of transmitter release (Herreras et al., 1988), depolarisation of cells and a reduction in the inward presynaptic calcium current (Segal, 1989). Low concentrations of the AChR agonist carbachol induces a form of long-term potentiation in area CA1 of the hippocampus that is NMDAR

independent and can modulate the threshold for tetanic LTP induction (Auerbach and Segal, 1994). Behavioral data also supports a role for acetylcholine release in hippocampal function, synaptic plasticity and learning in which blockade of presynaptic muscarinic receptors (M2) improved performance in the Morris water maze (Quiron et al., 1995).

In the present study, extracellular application of the AChR agonist carbachol produced a significant reduction in the size of the fEPSP in both wild type 129/Sv and $fyn^{129/Sv-/-}$ slices. This reduction in fEPSP size was associated with an increase in the PPF ratio, indicating that the probability of transmitter release was altered by this manipulation. The changes in fEPSP slope were mirrored in both wild type 129/Sv and $fyn^{129/Sv-/-}$ slices at concentrations above 0.1μM. This indicates that the process by which mAChRs modulate presynaptic transmission (primarily by inhibition of N type calcium channels) was not affected by the fyn mutation.

6.8. Manipulation of release by adenosine receptor activation

Adenosine causes decreased glutamate release and hyperpolarisation of pyramidal neurones. These effects are mediated by N type VGCCs, the activation of a inward rectifying postsynaptic potassium channel conductance and actions of adenosine on the axonal propagation of action potentials (Dunwiddie and Haas, 1985, Wu and Saggau, 1994, Debanne et al., 1996). Presynaptic A_1 receptors exist in the hippocampus (Swanson et al., 1995), which inhibit the actions of adenylate cyclase and decrease the production of cAMP. *In vivo* adenosine release tonically inhibits presynaptic function at excitatory synapses but does not inhibit transmission at GABA-ergic synapses (Dunwiddie, 1985, Lambert and Teyler, 1991). The net effects of adenosine release is to depress excitatory transmitter release, reduce postsynaptic excitability whilst leaving inhibition unaffected.

2-chloro-adenosine (CADO) is a non-hydrolysable analogue of adenosine. Slices were exposed to CADO by bath application which produced a significant reduction

in fEPSP slope in both wild type 129/Sv and $fyn^{129/Sv-/-}$ animals. This effect was associated with an increase in PPF ratio, indicating a decrease in transmitter release. No effect of the fyn mutation was seen however. The reversal of these effects by DPCPX enhanced the fEPSP slope in both wild type and $fyn^{129/Sv-/-}$ slices to an equivalent level, approximately 40% above control response slope. This indicates that there is a tonic inhibition of transmitter release by adenosine that is equivalent in both groups of animals.

The loss of fyn appears to mediate a presynaptic increase in transmitter release as determined by the reduction in PPF ratio. However, several independent manipulations of presynaptic release through calcium, adenosine and mAChR receptors showed little or no involvement of fyn in these presynaptic pathways, all of which modulate the calcium dynamics of transmitter release. If fyn is not modulating PPF by means of presynaptic calcium dynamics, another possibility is that fyn is expressed presynaptically and is directly interacting with the presynaptic release machinery.

6.9. A presynaptic expression for fyn and possible mechanisms of action

Several tyrosine phosphoproteins are located in the presynaptic vesicles that contain neurotransmitter and the phosphorylation state of these proteins can mediate stages of the vesicle fusion and release process. Both synaptotagmin and synaptogyrin are tyrosine phosphorylated and found in synaptic vesicles. Biochemical analysis showed no difference in the phosphorylation state of these two proteins in fyn knockout animals (Figure 28) indicating that (at least in the case of these proteins) fyn is not the endogenous kinase responsible for their regulation. What, if any, is the other evidence for a presynaptic locus for fyn? Fyn is involved in several developmental stages of neurogenesis including myelination (Umemori et al., 1994, Umemori et al., 1999), nerve growth cone extension (Beggs et al., 1994), the organisation of the cytoskeleton (Thomas et al., 1995) and regulation of FAK, which is responsible for the stabilization of focal adhesion contacts to the extracellular

matrix (ECM, Beggs et al., 1997). Fyn, src and yes have also been associated with various growth factor receptors that are expressed in developing axons (Parsons and Parsons, 1997, Desai et al., 1997). These functions for fyn tyrosine kinase suggest a presynaptic location for fyn in the developing brain.

However, the expression of fyn in the chick neural retina is located primarily in the cell body in mature neurones. Levels of fyn protein expression seen in synaptic membrane fractions of developing and mature retinal neurones are similar, however there was a significant decrease (around 47 fold) in autophosphorylation activity of fyn in the synaptic plasma membrane fraction between the developing and adult stages (Ingraham et al., 1992). Synaptic vesicles contain several phosphotyrosine proteins, and fyn has been postulated as the kinase responsible for their regulation (Greengard et al., 1993, Pang et al., 1988). To date however the presence of fyn in synaptic vesicles has not been established. Another member of the same family of tyrosine kinases, src, has been located in the plasma membrane of synaptic vesicles (Benfenati et al., 1992) and has been shown to be responsible for 70% of the tyrosine phosphorylation seen in synaptic vesicles and the phosphorylation of other synaptic vesicle proteins e.g. synaptophysin (Maness et al., 1988, Pang et al., 1988, Barnekow et al., 1990). Considering the unchanged state of two key presynaptic vesicle tyrosine phosphoproteins in $fyn^{129/Sv-/-}$ animals, the lack of reports relating to fyn expression in synaptic vesicles, the lack of effect of fyn on presynaptic modulations and the presence of another tyrosine kinase (src) and its known substrates indicates that fyn does not play an essential role in presynaptic tyrosine phosphorylation involved with neurotransmitter release in adult neurones. This raises the possibility that fyn mediates a change in PPF ratio by a postsynaptic mechanism.

Various studies at the neuromuscular junction and squid giant synapse, including changes in Ca^{2+} / Mg^{2+} ratios, quantal analysis and measurements of presynaptic calcium levels and currents, have suggested that the mechanisms responsible for PPF are presynaptic in origin (Charlton et al., 1982, Katz and Miledi, 1967, 1968, Llinas et al., 1981). The residual calcium hypothesis is often applied to synaptic transmission in the CNS to explain PPF (Creager et al., 1980, Manabe et al., 1993,

McNaugton et al., 1982, Schulz et al., 1994) although evidence suggests that alternative mechanisms may also be involved (Chapman et al., 1995, Winslow et al., 1994, Wang and Kelly, 1996, 1997, Nathan et al., 1990, Nathan and Lambert, 1991). The assumptions made in this hypothesis are that the level of transmitter release is dependent on the level of intracellular calcium, the probability of release at any given site and the number of releasable sites. It does not take into account such presynaptic factors as saturation of transmitter release, changes in vesicle refilling and docking, the effect of calcium influx on the presynaptic axon excitability (Stevens and Wang, 1995) or modulation of postsynaptic receptor functions. For example if the level of postsynaptic receptor desensitization changes between the first and second responses, the level of the secondary response may be altered in a manner independent of transmitter release. The concept of changes in PPF values being indicative of a change in presynaptic release is an oversimplification, and recent evidence supports this case.

Postsynaptic manipulations that affect PPF have been reported. Postsynaptic injections of BAPTA (a calcium ion chelator) or $CaMKII_{281-302}$ (an inhibitor peptide of CaMKII) both block the decrease in PPF ratio and the increase in EPSP / EPSC amplitude associated with increasing the extracellular calcium concentrations. This implies that the facilitation of neurotransmitter release by extracellular increases in calcium concentration is mediated in part by postsynaptic mechanisms. Direct injections of calcium (Ca^{2+}) / calmodulin (CaM) to the postsynaptic cell via a patch electrode decrease the ratio of PPF, the effects of which were prevented by inhibitors of CaMKII and PKC. The crucial finding of these studies is that cyclothiazide (CYZ, an inhibitor of AMPAR desensitisation) can increase PPF ratio, synaptic potentials and can reverse the decrease in PPF ratio brought about by Ca^{2+} / CaM. The level of AMPAR desensitization can therefore modulate the magnitude of PPF in the hippocampus (Wang and Kelly, 1996, Wang and Kelly, 1997). The level of GABA mediated inhibition has been noted to mediate the level of PPF in the CA1 region of the hippocampus, by depression of the $GABA_B$ mediated IPSP (Nathan et al., 1990, Nathan and Lambert, 1991). Using a cell membrane permeable calcium chelator (BAPTA-AM) to limit intracellular calcium elevation whilst modulating the

extracellular concentration of calcium, no effect on the level of PPF was seen, indicating that intracellular calcium concentrations are not the only factor involved (Winslow et al., 1994). It should be noted that the postsynaptic modification of PPF does not conflict with the residual calcium hypothesis, but requires that the analysis of results from this type of study should take into consideration all possible factors which may affect the facilitation level, including the sensitivity of the postsynaptic receptors.

Fyn knockout mice could display an increase in transmitter release combined with a modification to postsynaptic receptor sensitivity. In a recent publication, evidence that the number of AMPA receptors is decreased in mice lacking fyn tyrosine kinase is presented (Narisawa-Saito et al., 1999). This raises the possibility that the smaller facilitation seen in fyn knockouts is due to the saturation of the postsynaptic receptor field on the second stimuli. If the number of functional AMPA receptors is limited in fyn knockout mice, the amount of transmitter released by a single stimulus may nearly saturate the postsynaptic response. If under conditions where paired or high frequency stimulation occurs, and the level of presynaptic release is increased, this may saturate the postsynaptic response, and limit the level of facilitation seen in both long and short-term plasticity in fyn knockout animals. In the case of wild type animals where presumably the number of AMPA receptors exceeds the amount of transmitter released, then this saturation effect will not limit any facilitation seen.

In relation to the impaired LTP seen in fyn mutants, the apparent increased probability of transmitter release could provide an alternative explanation for this phenotype. The probability of presynaptic transmitter release is a finite variable, ranging from zero (failure of release) to unity (release on every occasion). Some evidence exists that the induction of LTP requires an increase in the probability of transmitter release. Synapses that have a low initial release probability can up-regulate the release to a greater extent than synapses with a high initial probability and thus show a greater level of facilitation (Schulz, 1994, 1997). In the case of fyn knockouts, which can be interpreted as a significant increase in release probability (as indicated from the decreased PPF ratio) the amount of available potentiation

could be restricted compared to wild type animals. Thus in conditions where high frequency stimulation is used to induce LTP, as in this and other studies on fyn knockouts (Grant et al., 1992, Kojima et al., 1997), this mechanism may limit the presynaptic release and subsequent postsynaptic depolarisation and calcium influx, showing impaired LTP. The only caveat to this explanation is that fyn LTP is strain dependent, whereas PPF is not.

The possibility exists that fyn is mediating a presynaptic change via its action on a retrograde messenger, although no direct evidence is available regarding this matter. It also possible that the tyrosine kinase cascades mediated by fyn and other src family members could interact with the CaMKII pathway, mediating a change in PPF through this mechanism. Further studies are required to elucidate the mechanisms of action. An ideal approach would be to use the whole cell attached recording technique to measure the rate of spontaneous mEPSP release in both wild type and fyn mutants. If there is an increased probability of release in fyn knockout mice, this could be detected in this manner. The use of the specific inhibitor PP2 could also be extended, if postsynaptic intracellular injection of this compound cause a modulation of PPF (in the same manner as CaMKII, see above and Wang and Kelly, 1997) then the locus of action could be determined.

Final remarks

It has been demonstrated in this thesis that the non-receptor tyrosine kinase fyn has important functions in the regulation of long-term potentiation, short-term facilitation and neuronal architecture in the hippocampal formation. The fact that one phenotype, impaired LTP, present in fyn knockout animals is strain specific indicates that the analysis of data from this type of genetic ablation requires great caution. In addition the continued use of highly inbred strains of animals for experimental purposes requires more attention to be focussed on the effects of the inbreeding and its relationship to phenotypes observed. The modulation of paired pulse facilitation by what appears to be a postsynaptically expressed protein indicates that the common assumption of a solely presynaptic mechanism for this short-term phenomenon must be re-evaluated to include possible postsynaptic modulations. There can be no question that src family tyrosine kinases are fundamentally involved in the regulation of synaptic transmission in the hippocampal formation, a brain structure that is essential for the development of new skills and the acquisition of knowledge. This warrants continued investigation into this family of kinases and other molecules which regulate the function of the NMDA receptor complex.

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Appendix – Publication

into an integrated percept[17], and it is these integrated object percepts that appear to be stored in visual working memory. Neurobiological accounts of working memory must therefore include a mechanism for keeping the features of an object bound together during the retention interval. A leading candidate mechanism is the use of oscillatory or temporally correlated firing patterns among the neurons that code the features of an object[18–20]. Such a mechanism can also readily explain the relatively small number of objects that can be held in working memory concurrently: as the number of concurrent objects increases, the possibility of accidental correlations between neurons that code different objects also increases[7]. However, this would not necessarily place any limits on the number of features that can be bound together into a single object representation, which is consistent with our findings. □

Methods

Ten neurologically normal college students participated in each experiment. Each of these observers received 32–40 trials in each condition, where a condition consisted of a combination of set size and some other variable, such as the presence or absence of a verbal load.

All stimulus arrays were presented within a 9.8° × 7.3° region on a video monitor with a grey background (8.2 cd m^{-2}), and the items in a given array were separated by at least 2.0 ° (centre to centre). One feature of one item in the test array was different from the corresponding item in the sample array on 50% of trials; the sample and test arrays were otherwise identical.

The experiments shown in Fig. 1a used sample arrays consisting of 1, 2, 3, 4, 8 or 12 coloured squares (0.65° × 0.65°), each of which was selected at random from a set of 7 highly discriminable colours (red, blue, violet, green, yellow, black and white). The experiments shown in Fig. 1b used the same stimuli, but set size was limited to 4, 8 or 12 items.

The experiments testing combinations of colour and orientation (Fig. 1c) used arrays of 0.93° × 1.15° rectangles, each of which was constructed by combining one of four orientations (vertical, horizontal, −45° and +45°) with one of four colours (red, green, blue and black). The stimuli used in the experiment shown in Fig. 1d were combinations of horizontal or vertical, red or green, small or large (0.13° × 1.0° or 0.13° × 2.0°) and continuous or broken (broken by a 0.26° black gap).

The colour–colour conjunction stimuli shown in Fig. 1e consisted of a small square (0.65° × 0.65°) embedded in a large square (1.3° × 1.3°). The inner and outer colours for a given object were selected from the set of red, green, violet and blue with the constraint that the inner and outer colours were always different from each other. The simple feature conditions of this experiment used either the large squares presented alone or the small squares presented alone.

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A role for the Ras signalling pathway in synaptic transmission and long-term memory

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Members of the Ras subfamily of small guanine-nucleotide-binding proteins are essential for controlling normal and malignant cell proliferation as well as cell differentiation. The neuronal-specific guanine-nucleotide-exchange factor, Ras-GRF/CDC25Mm (refs 2–4), induces Ras signalling in response to Ca^{2+} influx[5] and activation of G-protein-coupled receptors *in vitro*[6], suggesting that it plays a role in neurotransmission and plasticity *in vivo*[7]. Here we report that mice lacking Ras-GRF are impaired in the process of memory consolidation, as revealed by emotional conditioning tasks that require the function of the amygdala; learning and short-term memory are intact. Electrophysiological measurements in the basolateral amygdala reveal that long-term plasticity is abnormal in mutant mice. In contrast, Ras-GRF mutants do not reveal major deficits in spatial learning tasks such as the Morris water maze, a test that requires hippocampal function. Consistent with apparently normal hippocampal functions, Ras-GRF mutants show normal NMDA (*N*-methyl-D-aspartate) receptor-dependent long-term potentiation in this structure. These results implicate Ras-GRF signalling via the Ras/MAP kinase pathway in synaptic events leading to formation of long-term memories.

Several distinct mechanisms leading to Ras activation and initiation of the MAP kinase (MAPK) cascade have been elucidated[8]. Growth-factor receptors of the tyrosine kinase family activate Ras proteins by recruiting the ubiquitously expressed Sos exchange

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factors to the cell membrane through the adapter protein Grb2 (ref. 9). In neurons, an increase in intracellular calcium levels activates the Ras/MAPK pathway[10].

The exchange factor Ras-GRF (also called CDC25Mm) is exclusively expressed in neurons of the postnatal and adult central nervous system (CNS)[11] and is mainly localized in the synaptosomal fraction[12]. Following activation of muscarinic M1 and M2 receptors, Ras-GRF becomes phosphorylated and this increases its exchange activity[6]. Instead of presenting a Grb2-binding domain, Ras-GRF contains an ilimaquinone domain. When intracellular calcium is increased, this domain is necessary for binding to Ca^{2+}–calmodulin and for Ras-GRF-dependent activation of the Ras/MAPK pathway[5].

To examine the role of Ras-GRF in the activity of the adult brain, we inactivated the mouse gene using homologous recombination in embryonic stem (ES) cells, by replacing the most 5′ region encoding the exchange-factor catalytic domain with the phosphoglycerate kinase (PGK) promoter-driven neomycin cassette (Fig. 1a). Ras-GRF–/– mice are viable and fertile. To characterize the mutant mice at the molecular level, we generated amino-terminal-specific antibodies against Ras-GRF. Using NIH3T3 cells expressing full-length Ras-GRF in immunoprecipitation assays, we showed that commercially available anti-C-terminal and our anti-N-terminal antiserum specifically recognized a single band of relative molecular mass (M_r) 140K, corresponding to the Ras-GRF gene product (Fig. 1b). Detection of p140 was competable with the corresponding antigen, the PHP fragment of Ras-GRF, further demonstrating the specificity of the N-terminal antibodies. We performed western blot analysis on brain extracts using the two different antisera. The N-terminal antibodies specifically and exclusively recognized $p140^{Ras\text{-}GRF}$ only in mice carrying the wild-type allele, demonstrating that the introduced mutation resulted in the complete loss of the $p140^{Ras\text{-}GRF}$ gene product (Fig. 1c). The same loss of signal was also demonstrated using the C-terminal-specific antibodies.

Interestingly, some of the Ras-GRF+/– mice also lacked $p140^{Ras\text{-}GRF}$ and by western blot analysis were indistinguishable from Ras-GRF–/– mice (Fig. 1c). A new imprinted locus on mouse chromosome 9, corresponding to that of Ras-GRF, has been identified[13]. Our data on Ras-GRF heterozygotes confirmed the reported paternal allele-specific expression (data not shown). The biological significance of this phenomenon, however, is unknown.

Histological analysis was carried out on adult brains of wild-type and Ras-GRF mutant mice. Nissl-stained coronal sections of mutant mice did not show morphological abnormalities (Fig. 2a, b), despite the fact that Ras-GRF is widely expressed in many CNS structures, including hippocampus, cerebral cortex and thalamus, as shown by *in situ* hybridization analysis of wild-type animals (Fig. 2c). No expression of Ras-GRF messenger RNA was detected in brain sections derived from Ras-GRF–/– mice (Fig. 2d). To study specific subpopulations of neurons in more detail, we used as markers the calcium-binding proteins calbindin-D28K, parvalbumin and calretinin, which are thought to have roles in buffering intracellular calcium and are expressed in different subgroups of neurons[14]. Staining patterns were identical in Ras-GRF–/– and wild-type mice, indicating that these calcium-binding proteins were expressed at normal levels (data not shown). Based on the observed behavioural phenotype (see below), we analysed the amygdala in more detail (Fig. 2e, f). This structure also contains high levels of Ras-GRF mRNA and has a normal appearance in the mutants, based on Nissl staining (not shown) and calbindin immunoreactivity. No signs of neuronal atrophy were detected at high magnification (Fig. 2f, inset). In conclusion, our histological analysis did not reveal any major morphological defects in Ras-GRF mutant mice.

Because Ras-GRF-dependent signalling may have a role in synaptic transmission and plasticity, Ras-GRF–/– mice were subjected to behavioural tests. The two-way avoidance test is a measure for both conditioned learning and emotional response to aversive (noxious) stimuli[15]. In this test, mice are placed in a two-chamber box and taught to avoid a signalled electric shock (unconditioned stimulus) by running into the opposite compartment. The shock is preceded by a warning light (conditioned stimulus). The role of the amygdala has been clearly demonstrated for this test, as for other emotional learning tests[16]. As shown in Fig. 3a, wild-type mice gradually learned to avoid the electric shock with a 40% success rate by day 5 of the experiment. The performance of Ras-GRF mutant mice was much worse, showing a less than 10% success rate on the same day ($P < 0.0001$). Dose–response curves using increasing shock

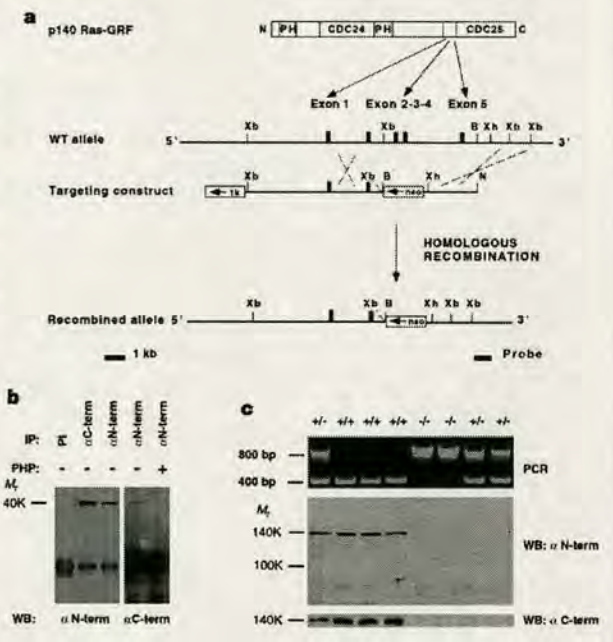

Figure 1 Generation of a targeted mutation in the mouse Ras-GRF gene. **a**, protein structure, genomic structure and targeting strategy. Location of the 2 pleckstrin homology (PH) domains, the CDC24-like domain and CDC25-like domain are indicated on the structure of $p140^{Ras\text{-}GRF}$. Five exons (designated 1-5) encoding amino-acid sequences N-terminal to the CDC25-like catalytic domain were mapped and sequenced in the murine wild-type Ras-GRF gene and are indicated as vertical bars. A 4-kb region of Ras-GRF containing exons 3-5 was replaced by the PGK promoter-driven neomycin cassette. This caused an increase of 2 kb of a diagnostic *Bam*HI fragment. Two different recombinant clones were used to generate mice carrying the Ras-GRF mutation which were subsequently used for the behavioural tests. Restriction sites: B, *Bam*HI; N, *Not*I; Xb, *Xba*I; Xh, *Xho*I. **b**, Characterization of new polyclonal antibodies against the N-terminal domain of Ras-GRF. Lysates of NIH3T3 cells ectopically expressing Ras-GRF were immunoprecipitated with preimmune serum (lane 1), anti Ras-GRF C-20 antibodies (Santa Cruz Biotech.) (lane 2), affinity-purified anti-N-terminal antibodies in the absence (lanes 3, 4) or presence of the purified PHP antigen (lane 5). After SDS-PAGE, blots were probed with affinity-purified N-terminal (lanes 1, 2, 3) or with C-20 (lanes 4, 5) antibodies. The specific 140K band is indicated. **c**, Biochemical analysis of Ras-GRF mutant mice. A litter of 1-month-old animals derived from a cross between two heterozygotes was first genotyped using polymerase chain reaction (PCR): 400 bp DNA fragment, wild-type allele; 800 bp DNA fragment, mutant allele. Brain extracts were prepared and subjected to western blot analysis with either anti-N-terminal or anti-C-terminal Ras-GRF-specific antisera. Positions of the 140K wild-type protein and of the 100K molecular marker are indicated.

intensities did not show differences between groups, ruling out a possible difference in shock sensitivity (data not shown). Other parameters such as presession activity were found to be normal ($P > 0.5$), indicating that the motor activity of both groups in the absence of conditioned stimulus was comparable (not shown). In addition, we have also tested a second Ras-GRF line derived from an independent ES clone targeted in the same way as above. This mutant strain also gave statistically significant impairment in avoidance learning (data not shown).

The one-trial inhibitory avoidance test makes use of the natural tendency of mice to move from an illuminated into a dark compartment[17]. Once the animal is in the dark compartment, it receives an electric shock. One single trial is normally sufficient for learning the task, which is to avoid the dark compartment during the probing trial (0.5 h to measure learning and short-term memory or 24 h to measure long-term memory). Note that this test requires no motor activity to manifest learning, in contrast to the two-way avoidance test. Both wild-type and Ras-GRF mutants clearly learn to avoid the dark compartment (Fig. 3b). The step-through latency time at 0.5 h increased in comparison to untrained mice ($P < 0.0001$), but no differences were detected between the groups ($P > 0.1$). In contrast, at 24 h, although wild-type mice persistently avoided the dark chamber, mutant mice seemed to have largely forgotten the task because they ran into the compartment with a latency intermediate between that of 0.5 h and untrained mice ($P < 0.0001$ between the two groups, at 24 h).

One-session fear conditioning can also be studied using a technique in which the foot shock used as an unconditioned stimulus is of higher intensity than in the shuttle-box, but only applied during a short period. When exposed to conditioned stimulus again, conditioned animals show an immobility ('freezing') reaction during which the animals refrain from all but respiratory movements. Freezing responses can be triggered with two different types of conditioned stimulus, each involving different brain structures[18]. In contextual conditioning, the conditioned stimulus is represented by the environment in which the unconditioned stimulus is delivered. This type of conditioning appears to depend on both hippocampal and amygdalar functions. In cued conditioning, the conditioned stimulus is a tone, and this type of conditioning is disrupted by lesions of the amygdala but not of the hippocampus. Mice were conditioned to tone and context during the same trial session and then tested separately, 0.5 and 24 h later. For measuring contextual learning, mice were placed in the same box where the training occurred and freezing was monitored for 2 min (Fig. 3c). At 0.5 h after conditioning, both wild-type and mutant mice showed the same freezing response, without significant difference ($P > 0.1$) but statistically higher than untrained mice ($P < 0.0001$). However, at 24 h, although wild-type mice still retained a strong freezing response, Ras-GRF mutants showed a dramatically reduced response ($P < 0.0001$). For measuring cued conditioning, mice were placed in a neutral cage for 1 min to minimize the contextual response, before delivering a continuous

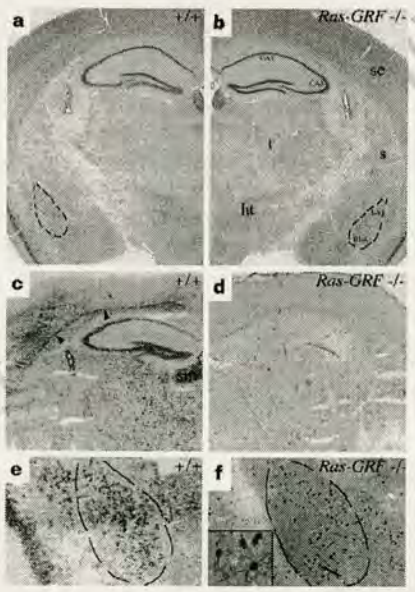

Figure 2 Ras-GRF mutant mice do not show gross morphological abnormalities in the brain. **a**, **b**, Coronal sections of wild-type and Ras-GRF mutant adult brains, stained with cresyl violet (Nissl). Note the normal appearance of the brain structures. sc, Somatosensory cortex; t, thalamus; s, striatum; ht, hypothalamus; LA, lateral nucleus of the amygdala; BLA, basolateral nucleus of the amygdala (stippled line). **c**, expression pattern of Ras-GRF mRNA by *in situ* hybridization. Note high expression levels in hippocampal structures and in the stria medullaris (sm), wide expression in the thalamus and in different cortical layers with more intense staining in a subpopulation of neurons located in layer VI (arrowheads). **d**, No expression of Ras-GRF was detected in the mutant section corresponding to **c**. **e**, Expression of Ras-GRF mRNA in the wild-type amygdala. **f**, Normal appearance of the lateral and basolateral nuclei of the amygdala of Ras-GRF mutant mice stained with calbindin-D28K. Inset in **f** is a higher magnification (neurons indicated with an arrow). Magnification: **a**–**d**, X25; **e**, **f**, ×200; inset, ×1,000.

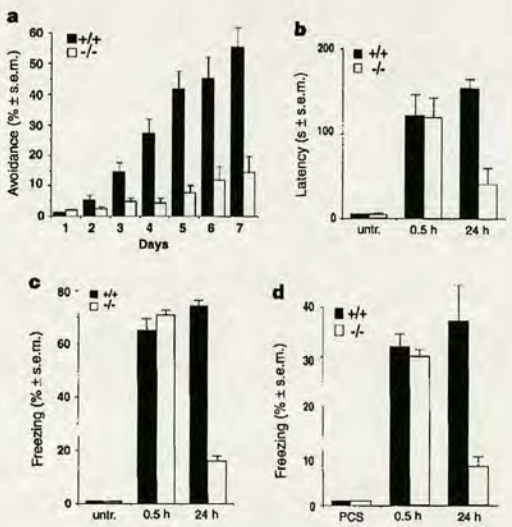

Figure 3 Impaired memory consolidation in Ras-GRF mutant mice during fear conditioning tests. **a**, Two-way avoidance learning test. Mice (wild-type, $n = 24$; mutants, $n = 26$) were trained for 7 days, 80 trials a day. Percentage of correct responses (avoidance of the shock) is shown. **b**, One-trial inhibitory avoidance test. Different groups of mice were tested for step-through latencies into the dark compartment: untr., untrained mice (wild-type $n = 18$; mutant $n = 18$), 0.5 h (wild-type $n = 8$; mutant $n = 8$) or 24 h (wild-type $n = 10$; mutant $n = 10$) after training. Mean step-through latencies expressed in s ± s.e.m. are indicated for both groups. **c**, Contextual fear conditioning test. Wild-type ($n = 12$) and mutant mice ($n = 10$) were tested before training (untr.), 0.5 h and 24 h after training for freezing (2 min) in the same training box. **d**, Cued fear conditioning test. The same mice used for contextual conditioning were subsequently tested 0.5 h and 24 h after training for freezing in the presence of a sound continuous for 1 min in a neutral cage, different from the training box, to minimize context-dependent freezing response. PCS is the pre-conditioned stimulus phase of 1 min. Cumulative percentages of freezing ± s.e.m. are indicated.

tone for 1 min (Fig. 3d). As observed for the one-trial inhibitory avoidance test and contextual fear conditioning, acquisition and short retention of the task appeared normal in the Ras-GRF−/− mice, whereas long-term memory was significantly diminished ($P < 0.0001$).

In conclusion, all these emotional learning paradigms clearly demonstrate that Ras-GRF−/− mice are severely impaired at the level of memory consolidation, rather than in the learning process itself.

In contrast to amygdala-mediated fear conditioning, spatial learning mainly depends on the function of the hippocampus[19]. To monitor spatial learning in rodents, animals were subjected to the Morris swimming navigation test[20]. In this test, the animal is placed in a pool and learns to find a submerged platform using visual cues outside the maze. After the intensive acquisition phase lasting 3 days, the platform is moved to the opposite position to test first for spatial learning of the former position (first trial, day 4) and subsequently for suppression of the old spatial information and reprogramming towards the new goal position (day 4 and 5). Clear learning curves were observed for the mutants ($P < 0.001$), with no differences in escape latency between mutant and control mice ($P > 0.1$) (Fig. 4a). Further evidence for normal spatial learning in both groups came from the retention test (Fig. 4b). During the first trial after the platform was moved to the opposite position, both groups showed preferential searching in the old goal quadrant relative to the others ($P < 0.0001$) but no differences between genotypes were seen ($P > 0.1$), indicated as the number of annulus quadrant crossings.

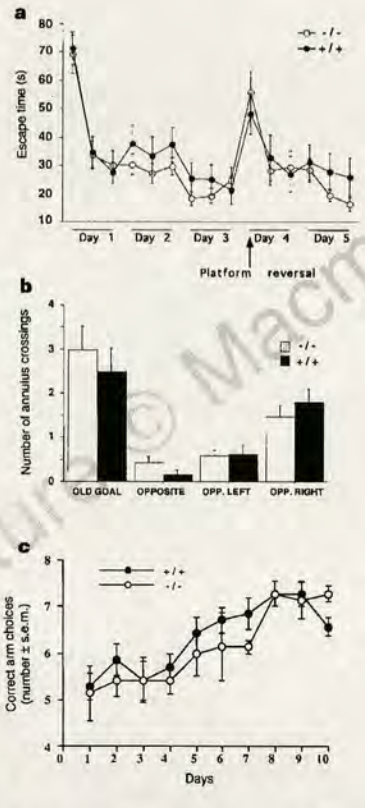

Figure 4 Hippocampal-dependent behaviour appears to be normal in Ras-GRF mutant mice. **a**, Spatial learning test. Wild-type ($n = 24$) and mutant mice ($n = 24$) were trained for 3 consecutive days (6 trials per day) with the submerged platform (acquisition phase) which was followed by 2 days of reversal phase, with the platform at the opposite position in the pool. Escape latency is expressed in seconds required to find the platform. **b**, Number of annulus crossings of the quadrants at the probing trial (day 4, trial 1). The value is indicated for all four quadrants: old goal, old training quadrant; opposite, opposite quadrant to the old goal position; opp. left, adjacent left quadrant to the old goal; opp. right, adjacent right quadrant to the old goal. **c**, Radial-maze test. Wild-type ($n = 14$) and mutant ($n = 14$) mice were trained for 10 consecutive days in an eight-arm radial maze. Learning performance is expressed in terms of the mean number of correct arm choices on each trial. The number observed at day 1 was taken as the starting point (5.28 ± 0.28 for wild-type; 5.14 ± 0.59 for mutants).

The radial-arm maze is another sensitive assay for hippocampal function. The advantage over the swimming test is that much less demanding motor activity is required to perform the task and different strategies can be used by the animal to explore the environment. Animals with hippocampal lesions are clearly impaired when tested on this task[21]. The performance of wild-type and mutant Ras-GRF mice was compared in a radial 8-arm maze with cues outside the maze. The procedure used allows the animals to visit each arm, searching for the food reward at any time. Variance analysis on the number of correct arm choices demonstrated that both groups of mice made significant progress in learning performance on the radial maze over the ten training sessions ($P < 0.0001$) (Fig. 4c). Both groups reached roughly the same level of performance and no difference between groups was apparent ($P > 0.1$). In addition, no differences in the strategy used to explore the arm were seen between the two groups (not shown). These data indicate that Ras-GRF mutants do not show major deficits in standard tests of hippocampal function.

To analyse several aspects of synaptic physiology and plasticity we performed electrophysiological experiments in the CA1 region of hippocampus and in the basolateral amygdala. In particular, we tested long-term potentiation (LTP), which is believed to be an important physiological event underlying learning and memory formation[22]. In CA1, stimulation of the Schaffer collateral pathway at 100 Hz in slices from Ras-GRF mutant and wild-type mice induced LTP of the synaptic response (Fig. 5a). This potentiation was not significantly different in slices from mutant mice (162 ± 14%; $n = 11$ slices, 5 animals) compared to slices from wild-type mice (158 ± 13.8%; $n = 13$ slices, 6 animals). The induction of LTP under these conditions was blocked when tetani were delivered in the presence of the NMDA receptor antagonist D-AP5 (50 μM) (data not shown). Extracellular field potential responses were recorded in the basolateral amygdala, which is known to support plasticity *in vitro*[23]. Delivery of 3 trains of stimulation of 10-burst theta frequency stimulation produced significant LTP (Fig. 5b, c) in +/+ controls (134 ± 12% of baseline; $n = 8$ mice, 16 slices), whereas −/− mice showed no such enhancement (104 ± 3% of baseline; $n = 7$ mice, 11 slices, $P < 0.001$). Although long-lasting plasticity was absent in −/− mice, there were no differences ($P = 0.78$) between the two groups in the first minute after tetanus (−/− 122 ± 3%; +/+ 124 ± 4%). When theta-burst tetanus was delivered to Schaffer collaterals in the CA1 region of hippocampal slices (taken from the same mice as the amygdala slices) LTP in −/− mice (130 ± 13% of baseline, $n = 6$ mice) was not different from LTP in +/+ mice (121 ± 10% of baseline, $n = 4$ mice, $P > 0.5$, Fig. 5b). Analysis of baseline synaptic response properties revealed that Ras-GRF−/− mice showed larger field excitatory post-synaptic potentials (EPSPs) in amygdala than +/+ mice (mean across intensities for −/− mice was 0.94 ± 0.04 V s^{-1}; $n = 9$ mice, 15 slices; for +/+ mice 0.54 ± 0.04, $n = 14$ mice, 25 slices) and in CA1 of hippocampus (mean across intensities for −/− mice was 2.55 ± 0.14 V s^{-1}; $n = 8$ mice, 10 slices; for +/+ mice, 1.44 ± 0.12; $n = 11$ mice, 14 slices), across a range of stimulus intensities (Fig. 5e). Analysis of variance indicates that the differences were significant in both structures ($P < 0.0001$). In the hippocampus, it is possible to use the amplitude of the presynaptic fibre volley to estimate the strength of afferent inputs, and thus

compare the synaptic input/output values more directly. Measurements of the ratio of the EPSP slope to the fibre volley amplitude (Fig. 5f) also indicated significant differences ($P < 0.0001$) between +/+ mice (mean ratio = 2.27 ± 0.23; $n = 11$ mice, 13 slices) and Ras-GRF–/– mice (mean ratio = 3.96 ± 0.27; $n = 8$ mice, 10 slices). Finally, we examined paired-pulse facilitation (Fig. 5d), a form of short-lasting plasticity that depends on presynaptic mechanisms[24]. In hippocampus, wild-type mice show paired-pulse facilitation values (1.38 ± 0.07) that were statistically indistinguishable from Ras-GRF mutant slices (1.30 ± 0.02, $P > 0.5$). The same was observed for amygdala slices (for +/+, 1.27 ± 0.05; for –/–, 1.19 ± 0.03, $P > 0.1$), suggesting that Ras-GRF is not absolutely necessary for this type of plasticity.

We have shown that Ras-GRF mutant mice have an impairment in the process of memory consolidation during fear-related behavioural tasks and electrophysiological impairments in the amygdala, a critical part of the neural circuitry involved in emotional responses. The role of the amygdala in regulation of the behavioural response to external and noxious stimuli has been clearly demonstrated[25,26]. In rats, lesions in the amygdala dramatically affect acquisition of both cued and contextual fear conditioning, whereas specific lesions at the level of the hippocampus affect only contextual fear conditioning[18]. In contrast, Ras-GRF mutant mice do not show deficits in the learning process itself, implying that Ras-GRF signalling seems to be specifically involved in the consolidation of long-term memory.

Although Ras-GRF is highly expressed in the CA1 region of the rodent hippocampus, we did not detect, using several standard protocols, any major abnormalities in hippocampal synaptic plasticity or hippocampus-dependent forms of learning. We cannot infer that hippocampal functions are completely normal, as there are differences in some aspects of synaptic transmission, and there may be changes in other forms of plasticity. In any case, Ras-GRF does not seem absolutely required for spatial learning. Alternatively, some compensatory events might have occurred in the Ras-GRF mutants to mask the hippocampal phenotype.

Currently there is little information on the mechanisms by which Ras-GRF might be involved in processes leading to changes in synaptic transmission and LTP. We favour the idea that Ras-GRF signalling is directly involved in synaptic plasticity, although an indirect effect on the activity of certain types of neurons in the amygdala cannot formally be excluded. The differential effects on synaptic plasticity between amygdala and hippocampus may reflect the ability of Ras-GRF to couple with distinct signal transduction mechanisms that predominate in each of these structures. LTP in the CA1 region of the hippocampus requires calcium influx via the NMDA receptor, in contrast to at least some important pathways in the basolateral amygdala in which LTP is NMDA receptor-independent[27]. Moreover, muscarinic receptors are highly expressed in the basolateral amygdala and muscarinic antagonists block LTP in this structure[28]. Because muscarinic receptors activate Ras-GRF, producing an increase in its phosphorylation[6], this pathway may be physiologically relevant to amygdala function. Thus Ras-GRF may be important in synapses where metabotropic receptors dominate synaptic plasticity and less important in those regulated by NMDA receptors.

Knockout experiments have demonstrated a role in long-term plasticity and behaviour for a number of protein kinase cascades[29]. Our finding that the Ras/Raf/MAP kinases pathway controlled by Ras-GRF is also involved in such processes strengthens the notion that multiple signalling events are simultaneously required for the generation of long-lasting synaptic changes, leading to consolidation of learning and memory processes. □

Methods

Generation of targeted mice. To construct the Ras-GRF targeting vector, a 1.3 kb *Xho*I–*Not*I and 5.6 kb *Xba*I–*Xba*I DNA fragments of cloned 129 strain Ras-GRF genomic DNA were used. After homologous recombination, a neomycin-resistance cassette was inserted while deleting 4 kb of the Ras-GRF locus, including exons 3–5 coding for the 5′ portion of the CDC25-like catalytic domain (nucleotides 2,982–3,260 of the published cDNA sequence)[3]. Germline-transmitting chimaeras from two recombinant cell lines were

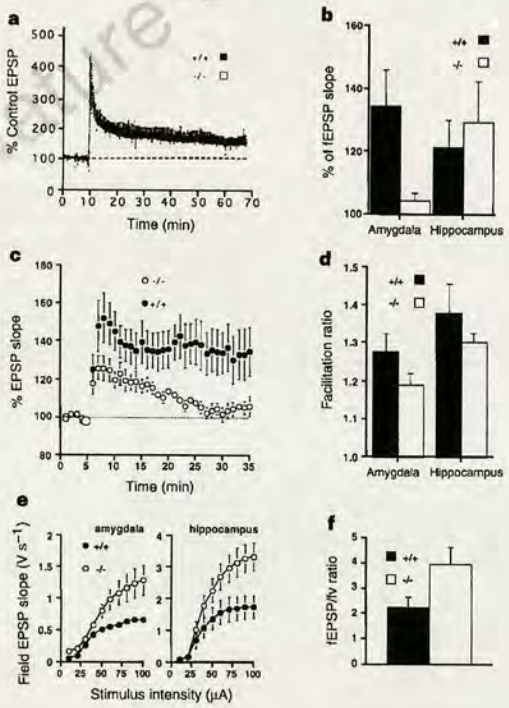

Figure 5 Impaired synaptic plasticity in the amygdala of Ras-GRF–/– mice. **a**, LTP in the hippocampus is unaffected by the Ras-GRF mutation following 2 trains of 100 stimuli each at 100 Hz. **b**, In both control and mutant hippocampal slices, theta-burst stimulation produces significant potentiation. **b**, **c** In +/+ amygdala slices, theta-burst stimulation is also an extremely effective protocol for LTP induction, but amygdala slices taken from –/– mice do not demonstrate significant potentiation 30 min after tetanus. **d**, Paired pulse facilitation is not different between wild-type and Ras-GRF–/– mice, in either the hippocampus or the amygdala. The indicated data are the average across the 30–100 μA range of stimulus intensities, at 30-ms interpulse intervals. **e**, Synaptic responses are significantly elevated in both amygdala slices (left) and hippocampal slices (right) taken from Ras-GRF–/– mice, compared to +/+ controls. **f**, After controlling for variability in afferent input strength by measuring the ratio of the field EPSP slope to the fibre volley amplitude in hippocampal slices, the difference between +/+ and –/– mice was still significant.

obtained by standard injection into C57BL/6 blastocysts, and the mutation was crossed into either 129/Sv or C57BL/6 genetic backgrounds.

Biochemistry. The Ras-GRF N-terminal fragment (PHP) corresponding to residues 1–149 of the published sequence[3] was used to raise specific antibodies. Total adult brain protein lysates were subjected to western blot analysis using polyclonal antibodies directed either against the N-terminal or the C-terminal portion of $p140^{Ras\text{-}GRF}$. Blots were developed using the ECL method.

Histology. For immunohistochemistry, 40-μm coronal cryosections were prepared and further processed using standard techniques. For *in situ* hybridization analysis, 16-μm sections were prepared and processed following the manufacturer's protocol for digoxigenin-labelled oligonucleotide probes (Boehringer Mannheim). The DNA fragment used as a probe corresponds to 3′ sequences of the published cDNA sequence of mouse Ras-GRF (nucleotides 1,915–4,174).

Behavioural tests. The mice used for all the behavioural tests were littermates of 9–16 weeks of age, kept on a 1 : 1 mixed genetic background between 129/Sv and C57BL/6. All the behavioural tests were performed as previously described[15,17,20,21,30]. In brief, for the two-way avoidance test, mice were placed in sound-proof shuttle-boxes (Campden Instruments) operated by a computer. After 2 min during which the mice were left undisturbed (pre-session), a conditioning light stimulus lasting 5 s was delivered and followed by a 10 s electric shock of 0.15 mA. Intertrial interval varied between 5 and 15 s. The animals underwent 80 trials a day for 7 days.

For the one-trial inhibitory avoidance test, mice were trained on an apparatus in which a straight alley was divided into two compartments. The smaller compartment was made of white Plexiglas. The larger one was made of black Plexiglass and was equipped with a removable cover of the same material to allow the compartment to be in darkness. A tensor lamp illuminated the small compartment. The floor of the larger compartment consisted of two oblique stainless steel plates folded at the bottom through which a constant current could be delivered. On the training day each mouse was placed in the lit compartment, facing away from the dark compartment. When the mouse had stepped with all four paws into the dark side, the door was closed, the step-through latency was recorded, and two foot shocks (0.4 mA, 50 Hz, 2 s) were delivered with an interval of 5 s. The maximum initial step-through latency allowed as a criterion for the animals entering the trial was 15 s. The mouse was then removed from the apparatus and returned to its home cage. Retention was tested 0.5 or 24 h later following a similar procedure, except that no shock was administered. A maximum step-through latency of 180 s was allowed in the test session.

For both contextual and cued fear conditioning, mice were trained within the same session, with the following protocol: the pretrial time of 1 min in the conditioning box (the same used for shuttle box) was followed by 15 s tone (conditioned stimulus, 3,000 Hz, 80 dB). During the last 5 s of tone a foot shock of 0.75 mA was delivered and after 15 s the procedure was repeated 5 times. During the training period, acquisition of the freezing response was monitored and no differences were found between the two groups. The freezing response to conditioned stimulus was monitored 0.5 h and 24 h after training for both tests: for contextual conditioning, mice were monitored for freezing for 2 min in the box used for training. For cued conditioning, mice were placed in a new, neutral cage, and freezing was monitored for 1 min in the absence of sound (pre-conditioned stimulus freezing) and for 1 min in the presence of a continuous sound (conditioned stimulus freezing). In both tests freezing was scored every 5 s.

The Morris navigation test was carried out in an open-field water maze of 1.5 m in diameter and filled with opaque water at the temperature of 25 ± 1 °C, located in a laboratory that contained prominent extra-maze cues. A hidden 15-cm-diameter platform was used. Trials lasted a maximum of 120 s. Spatial training consisted of 18 trials (6 per day) during which the platform was left in the same position. After 3 days of learning, the platform was moved to the opposite position and reversal learning was monitored for 2 additional days (6 trials per day).

For the radial maze test the apparatus was a grey plastic maze with eight identical arms radiating form an octagonal starting platform (perimeter, 7 × 8 cm). On each training trial a 20-mg food pellet was placed at the end of each arm and the animal was placed facing a randomly selected direction on the central platform. Animals received one trial per day; each daily trial terminated when eight choices were made or 15 min had elapsed. An arm choice was defined as placement of all paws on a maze arm. An error was committed when an animal enters a previously visited arm.

Electrophysiology. Slices of amygdala and hippocampus were prepared using standard methods and media. Brains were removed to ice-cold artificial cerebrospinal fluid (ACSF, 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 11 mM glucose), cut on a Vibratome or a gravity tissue chopper at 400 μm, and maintained in a submersion-type chamber at 28–32 °C. Extracellular field potentials were recorded using glass pipettes filled with 1 M NaCl or carbon-fibre electrodes placed in the basolateral amygdala or in stratum radiatum of hippocampal CA1. CA1 responses were evoked by stimulation of the Schaffer collaterals using bipolar or monopolar stainless steel electrodes, and responses in the basolateral amygdala were elicited by stimulation of the lateral amygdala with monopolar stainless steel electrodes.

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